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CHARACTERIZATION OF MYELOPEROXIDASE FROM BOVINE SPLEEN: ROLE OF ASCORBIC ACID IN MYELOPEROXIDASE FUNCTION

by

LEAH ALMACEN MARQUEZ

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

SPRING 1990

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THE UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled "Characterization of Myeloperoxidase from Bovine Spleen: Role of Ascorbic acid in Myeloperoxidase Function" submitted by Leah Almacen Marquez in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

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Date Dec. 12, 1989

This thesis is dedicated to my dear parents.

ABSTRACT

The green heme protein isolated from bovine spleen was found to be analogous to myeloperoxidase. A comparison of spectroscopic properties suggests that their chromophores, enzyme intermediates and cyanide binding properties are very similar.

Spectral results of cyanide binding to canine myeloperoxidase support the presence of two heme binding sites; an isosbestic point at 444 nm and a linear Scatchard plot suggest that the binding affinity of cyanide to the two subunits of the enzyme is the same. The dissociation constant is 0.53 μM. The pH dependence of the apparent second order rate constant indicates the presence of an acid-base group on the enzyme with a pK_a of 3.8 ± 0.1. The protonated form of cyanide binds to the basic enzyme with a rate constant of (4.3 ± 0.3) X 10^6 $M^{-1}s^{-1}$.

The optical spectra of relatively pure compounds I and II of myeloperoxidase from bovine spleen have been obtained using a 20-fold excess of H_2O_2. The apparent second order rate constant for compound I formation is (1.5 ± 0.1) X 10^7 $M^{-1}s^{-1}$ while that for compound II formation is (8.1 ± 0.1) X 10^4 $M^{-1}s^{-1}$.

Ascorbic acid stimulates myeloperoxidase activity by converting inactive compounds II and III to the native enzyme. A comparative spectral and transient state kinetic study was done on compound II of myeloperoxidase from three sources namely, human neutrophils, canine pus and bovine spleen, and their reaction with ascorbate. The reaction of all three compounds II involves a simple binding interaction before enzyme reduction with an apparent dissociation constant of $6.3 \pm 0.9 \times 10^{-4}$ to $2.0 \pm 0.3 \times 10^{-3}$ M and a first order rate constant for reduction of 12.6 ± 0.6 to $18.8 \pm 1.3\ s^{-1}$. The optimum pH is 4.5 and at this pH the activation energy for the reaction is 13.2 kJ mol^{-1}. Chloride cannot reduce compound II whereas iodide reduces compound II to native enzyme at a rate comparable to that of ascorbate. This explains why ascorbate accelerates chlorination but inhibits iodination.

A relatively pure and stable compound III of bovine spleen myeloperoxidase was also prepared by using the oxidation of dihydroxyfumarate to generate $O_2^{-\cdot}$. Compound III decayed to native enzyme without any detectable intermediates. Ascorbic acid reduces compound III to native form with a second order rate constant of $(4.0 \pm 0.1) \times 10^2\ M^{-1}s^{-1}$.

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CHAPTER ONE

INTRODUCTION

1.1. PEROXIDASE ENZYMOLOGY

1.1.1. Definition and Some Examples

Peroxidases are enzymes that catalyze the oxidation of a wide spectrum of substrates by hydrogen peroxide or other hydroperoxides. They are widely distributed throughout plants, animals and microorganisms and carry out a variety of biosynthetic and degradative functions (1).

The structures and reaction mechanisms of some plant and microbial enzymes have been well-studied. Horseradish peroxidase (2), turnip peroxidase (3) cytochrome c peroxidase (4), lignin peroxidase (5), manganese peroxidase (6), chloroperoxidase (7) and bromoperoxidase (8) are some of these enzymes which have gained an extensive research interest. Their availability in highly purified form, the stability of the enzyme intermediates and the intense environmentally-sensitive Soret absorbance band greatly facilitated such studies. Several animal peroxidases have also been isolated and studied to date: lactoperoxidase (9), thyroid peroxidase (10), eosinophil peroxidase (11), myeloperoxidase

(12) and salivary peroxidase (13).

1.1.2. General Structure and Enzyme Intermediates

Generally, peroxidases consist of a heme and glycoprotein. The heme prosthetic group of most peroxidases is ferriprotoporphyrin IX (Fig. 1.1).

In the normal catalytic cycle, the ferric enzyme first reacts with a hydroperoxide to form compound I, which has two oxidizing equivalents more than the ferric enzyme. The metal center, Fe[III], activates the heterolytic cleavage of the hydroperoxide and stores one oxidizing equivalent and the porphyrin ring stores the other oxidizing equivalent (14). In most cases, compound I undergoes a one-electron reduction by a hydrogen donor (AH_2) to give compound II and a free radical, followed by a further one-electron reduction back to the native enzyme. Compound I can also undergo a two-electron reduction to give the native enzyme (15,16).

The basic mechanism can be represented as follows:

[1.1] $\text{Peroxidase} + H_2O_2 \longrightarrow \text{Compound I}$

[1.2] $\text{Compound I} + AH_2 \longrightarrow \text{Compound II} + {}^{\cdot}AH$

[1.3] $\text{Compound II} + AH_2 \longrightarrow \text{Peroxidase} + {}^{\cdot}AH$

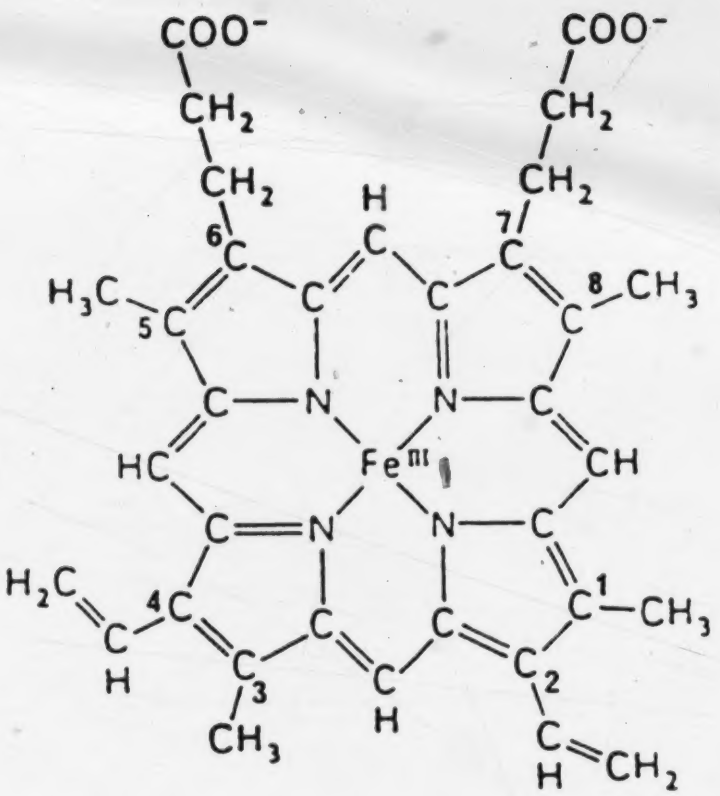

Figure 1.1. Structure of ferriprotoporphyrin IX : the prosthetic group of most peroxidases. The intense environmentally sensitive Soret absorbance at about 400 nm is due to the conjugated tetrapyrroles.

It is generally accepted that the structures of the intermediates are independent of the oxidizing and reducing substrates used to form them.

1.2. MYELOPEROXIDASE

Myeloperoxidase (EC 1.11.1.7) was first isolated and characterized by Agner in 1941, although as early as 1868 pus was found to exhibit peroxidatic activity (17). The presence of the enzyme in the white blood cells as well as its detoxifying ability provided an indication of its possible involvement in the body's defense mechanism against infection.

1.2.1. Localization

The white blood cells or leucocytes can be divided into nongranular and granular forms. The latter, also called granulocytes, are characterized by a many-lobed nucleus, hence the term polymorphonuclear (PMN) leucocytes. Certain leucocytes are capable of phagocytosis: they include the PMN leucocytes as well as the monocytes. Although monocytes are generally considered to fall in the nongranular category, they do in fact contain small numbers of granules. Myeloperoxidase is localized in these granules (12).

1.2.2. Cellular Targets

Phagocytic leucocytes respond to microbial invasion by a complex series of events which culminates in the ingestion into a vacuole and destruction of the invading microorganisms. Associated with these events is the secretion of toxic agents into the vacuole. Among the potentially toxic agents is the myeloperoxidase-H_2O_2-halide system. In the presence of H_2O_2 and halide, chiefly Cl^-, *in vivo* , myeloperoxidase catalyzes the two-electron oxidation of Cl^- leading to the formation of HOCl which is a very strong oxidizing agent. The isolated system has been found to be toxic to a variety of bacterial and fungal strains (18-20).

Myeloperoxidase also shows *in vitro* cytotoxic activity against cellular targets such as platelets (21) and spermatozoa (22). A number of biomolecules have also been shown to be modified by myeloperoxidase (23-27). It is assumed that most of these molecular alterations are either directly or indirectly detrimental to cell survival. Recently, it was reported that myeloperoxidase-derived oxidants inactivate proteinases and other microbicidal enzymes. Although inactivation of the cell's granule enzymes may seem to be self-defeating, it may be beneficial to the host since it would minimize the release of potentially harmful

hydrolytic proteins into the tissues (28). However, the products of the MPO system have also been found to contribute to tissue damage associated with rheumatoid arthritis (29,30).

1.2.3. Mechanisms of cytotoxicity

There have been several mechanisms proposed for the cytotoxicity of the myeloperoxidase system. A sharp increase in oxygen consumption was always found to accompany phagocytosis. It is generally agreed that O_2 is first reduced to $O_2^{\cdot -}$ which is then converted to H_2O_2 and O_2 (12). $O_2^{\cdot -}$ and H_2O_2 may have microbicidal properties; moreover, these species are known to be precursors of more potent oxidizing agents.

Myeloperoxidase catalyzes the oxidation of Cl^- ion by H_2O_2 to give HOCl (31,32). HOCl is highly reactive, being able to oxidize many biological molecules, especially those with sulfhydryl groups, and in this way is considered to exert its bactericidal action (33). Moreover, HOCl is known to react with H_2O_2 to form singlet oxygen which has also been proposed as a species probably responsible for the enzyme's microbicidal activity (34-36).

Some workers have also suggested that the enzyme's cytotoxic

action is due to its ability to deaminate and decarboxylate appropriate substances to aldehydes (37) or to form chloramines (38-40). Whichever species is involved in the microbicidal activity of myeloperoxidase it is apparent that the first oxidation product formed is HOCl. HOCl in itself is a strong oxidizing agent but it can also be a precursor of more toxic agents.

1.2.4. Structure and Properties

The investigation of the structure and physicochemical properties of myeloperoxidase is of primary importance for an understanding of the physiological role and function of the enzyme. Myeloperoxidase is unique with respect to other peroxidases in the complexity of its structure and difference in heme type.

Myeloperoxidase contains two atoms of iron per molecule (41-43). Several groups have demonstrated that myeloperoxidase is a single molecular species composed of two heavy and two light subunits (44-47). However, the literature is plagued with marked differences in the molecular weight (MW) as well as the nature of the subunits. The reported MW of myeloperoxidase isolated from different species and determined by

various methods range from 130 to 153 kDa (41-50). There have also been contradictory reports as to the number and molecular weights of the subunits of the enzyme (48, 51,52). One of the most significant findings about the structure of myeloperoxidase arose from the reductive cleavage of the enzyme in half, producing enzymatically active hemi-myeloperoxidase (53,54). This supported the proposal that the enzyme has an $\alpha_2\beta_2$ quaternary structure. More recently, x-ray diffraction studies suggested a model for the molecular packing of the myeloperoxidase molecule which consisted of two identical or near-identical halves related by a two-fold symmetry axis (55).

In the native enzyme the visible and Soret absorption are considerably red-shifted compared with those peroxidases containing porphyrin as prosthetic group. Recent resonance Raman scattering studies have shown that the chromophore of myeloperoxidase is a chlorin or dihydroporphyrin (Fig. 1.2) (56-59).

Myeloperoxidase exhibits an amazing degree of stability over the other heme-containing peroxidases. Addition of acidified acetone to these peroxidases causes the release of the prosthetic group and the precipitation of the glycoprotein component. However, when acidified acetone

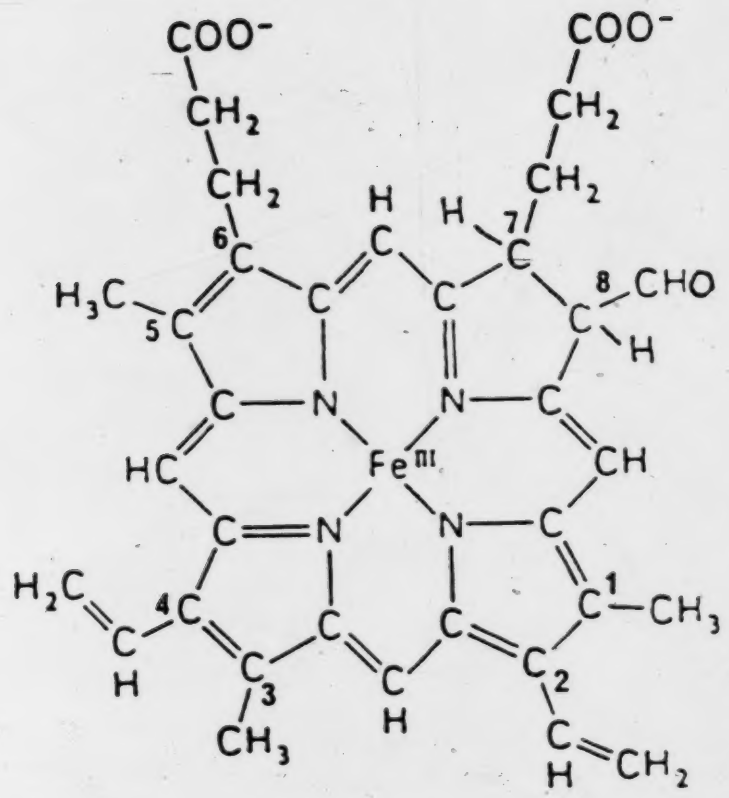

Figure 1.2. Structure of chlorin: the prosthetic group of myeloperoxidase. The methyl group in C-8 has been replaced by a formyl group and the ring has been reduced in relation to ferriprotoporphyrin IX (Fig. 1.1).

is added to myeloperoxidase, the whole molecule precipitates without breaking the linkage between prosthetic group and glycoprotein (60). Moreover, the enzyme is reported to retain 100% of its activity at extreme p(2.45-12.8) and even at 60 °C (61).

The stability of the enzyme can partly be accounted for by the extensive disulfide bridging in the molecule. It has been found that myeloperoxidase has a very high cysteine content (51). The amino acid sequence has been reported (62,63), as well as the carbohydrate content (48).

1.2.5. Biosynthesis

There have also been a number of studies on the biosynthesis of myeloperoxidase. Investigations of this type are aided by the use of HL-60 promyelocytic leukemia cells which express this enzyme (50, 64). The aim of such studies is to gain insight into the hereditary myeloperoxidase deficiency which has been identified as a relatively common defect. *In vitro* translation studies demonstrate that myeloperoxidase is synthesized as a 75-80 kDa polypeptide. Synthesis begins at the myeloblast stage of development and ceases as the cells differentiate to granulocytes and macrophages (62,65). It has been reported that

myeloperoxidase deficiency is due, not to the absence of the gene for myeloperoxidase synthesis, but to a defect in cotranslational or posttranslational processing of the precursor protein of myeloperoxidase, resulting in a failure to package the enzyme correctly into the granules (64).

1.2.6. Other Functions

Aside from the direct contributions myeloperoxidase makes to the defense mechanism of mammals it was found to be involved in other protective functions as well. Myeloperoxidase has recently been reported to inhibit DNA strand breakage induced by oxygen radicals. The enzyme was found to have DNA binding properties which may protect the genetic material of myeloid cells from damage inflicted by oxygen radicals produced during myeloid cell maturation and function (66).

On the other hand, myeloperoxidase was implicated as a modulator of the inflammatory response. Apparently, HOCl produced by the myeloperoxidase system activates collagenase which is involved in the degradation of collagen. The destruction of collagen plays a key role in the development of inflammatory disease states affecting every organ system in the human body (67). Thus, there are both positive and negative

biological consequences that follow from the chemical reactivity of the reaction products of this enzyme (68).

1.2.7. Applications

Other peroxidases with halogenating properties have found their practical uses as synthetic tools (69), analytical diagnostics (70,71) and in pollution control (72,73).

Recognizing the antibacterial properties of HOCl, myeloperoxidase has been used as *in situ* microbicidal-generating agent. In combination with iodide ions it has been formulated with powdered drug preparations to help preserve the drug from bacterial decomposition. One can also envision the possible use of myeloperoxidase as a therapeutic agent. If it can be delivered to the site of an infection or tumour (for example by coupling the enzyme to a monoclonal antibody) myeloperoxidase can release HOCl and selectively destroy the invading agent or cell (68).

The major obstacle to the commercial application of myeloperoxidase is the cost of producing the enzyme. Thus, finding an abundant source of the enzyme, employing a simple isolation and purification procedure as well as exploring ways to enhance its enzymatic activity will all have significant utility.

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CHAPTER TWO

PURIFICATION OF MYELOPEROXIDASE FROM BOVINE SPLEEN: CYANIDE BINDING PROPERTIES

2.1. INTRODUCTION

Myeloperoxidase (or verdoperoxidase as it was first called because of its green color) was first isolated by Agner in 1941 from the empyema fluid or sputum of tuberculosis patients (1). The enzyme is bound to the structure of white blood cells (leucocytes) and thus, by disintegration of the latter, myeloperoxidase can be released. The isolation of myeloperoxidase in quantities that will suffice for studies of the structure and properties of the enzyme required a starting material enriched in leucocytes. In 1958, Agner used infected dog uteri to obtain a crystalline preparation of myeloperoxidase (2). Canine pus thus became one of the richest source of myeloperoxidase (3). However, other starting materials have also been used including rat chloroma tissue (4); guinea pig bone marrow (5); human monocytes (6); human plasma (7); and HL-60 cells (8-10). Polymorphonuclear (PMN) leucocytes which contain about 5% by dry weight myeloperoxidase (11,12) are the most commonly used source of the enzyme (13-22).

Attempts to obtain sufficient quantities of the enzyme from outdated human blood for kinetic studies in our laboratory were unsuccessful. The major drawback was the limited amount of available blood. In 1981, a green heme protein was isolated from bovine spleen that was claimed to have properties similar to those of myeloperoxidase (23). As higher yields were reported bovine spleen was used as starting material for isolating the enzyme. In this work the procedure used for isolating the bovine spleen enzyme is described in detail as well as a preliminary characterization in terms of molecular weight determination and optical and cyanide-binding properties.

2.2. MATERIALS AND METHODS

The purification of myeloperoxidase from bovine spleen was carried out following a combination of published procedures (24-26) with details added and some minor modifications.

Beef spleens were obtained from Gainers, Inc., a meat-packing plant in Edmonton, Alberta. Fresh spleens were used for the enzyme isolation procedure since storage by freezing gives lower yields. The thick outer skin was trimmed off and most of the veins and other

connective tissues were removed.

Acid extraction. For a typical enzyme isolation, 1000 grams of macerated spleen were suspended in 2 liters of 0.25 M KCl and stirred occasionally at 4 °C for 30 minutes. The mixture was then homogenized in a Waring blender at maximum speed for 2 minutes. Foam which formed at the surface was scooped out. The pH of the spleen homogenate was adjusted to 3.5 by the dropwise addition of 6 M HCl. The acidic homogenate was stirred for 5-6 hours at 20°C then centrifuged at 10,400 X g (8,000 RPM) in a Sorvall- type GSA rotor with a Sorvall RC2-B centrifuge for 15 minutes. The supernatant (typical volume: 3 liters) was filtered through glass wool to remove small waxy particles. Citric acid (final concentration: 0.1 M) was then added to the clear supernatant. Following that, the pH of the solution was adjusted to 5.5 with 12% NaOH.

Batch Enzyme Adsorption. The supernatant was then treated with pre-cycled Whatman P11 cellulose phosphate fibrous cation exchanger and stirred for one hour. Precycling was done by stirring the cellulose phosphate into 25 volumes of 0.5 N NaOH and allowing to stand for 5 minutes. The supernatant was then decanted and the cellulose phosphate

washed several times until the pH of the filtrate was 11 or lower. The base-treated cellulose phosphate was then stirred into 25 volumes of 0.5 N HCl and the same procedure followed as before until the pH of the filtrate was above 3.0. The P11 cellulose phosphate which had adsorbed the enzyme was washed 2X with 100 ml water, and then resuspended in 100 mL of 2.0 M KCl and stirred for two hours at medium speed. The supernatant was decanted and the cellulose phosphate washed 2X with 25 mL 2.0 M KCl. The washing was pooled with the rest of the supernatant and centrifuged at 10,000 X g for 20 minutes.

Ion Exchange Chromatography. The resistance of the supernatant was measured using a conductivity cell and a Yellow Springs Instrument Model 31 conductivity bridge and compared to a KCl standard curve. The supernatant was concentrated to about 40 mL using a 200-mL capacity Amicon ultrafiltration apparatus equipped with an XM50 or PM30 Diaflo ultrafiltration membrane and an applied pressure of 50 psi. During this concentration step the ionic strength remained the same. This crude enzyme solution was then diluted with deionized water to an ionic strength of 0.1 M before loading to the chromatographic column. The column was 30 cm long with a 1.4 cm inner diameter. CM-Sepharose

CL-6B (Pharmacia) pre-swollen gel was suspended in 0.1 M phosphate buffer, pH 6.0 and deaerated for 30 minutes. The crude enzyme solution was then poured into the column and washed with over 3X its volume of the same buffer at a flow rate of 1 mL/min. The applied enzyme sample remained as a dark green band on top of the column. About 200 ml of 0.1 M phosphate buffer pH 6.0 was used to wash the sample at the same elution rate as before. A linear ionic strength-pH gradient elution was then started. The gradient apparatus used was a home-made Parr type (27) using 0.1 M phosphate buffer pH 6.0 as the starting buffer and 1.0 M phosphate pH 7.0 as the limiting buffer. The elution was done with the help of an LKB Microperpex peristaltic pump and a Pharmacia FRAC-100 programmable fraction collector set on a time-based mode of 10 min/ fraction.

Fractions with Rz (A_{430}/A_{280}) $\geq$ 0.4 and $\geq$ 0.7 were pooled separately. The first set of fractions were rechromatographed on the CM-Sepharose CL-6B column and fractions from this column with Rz $\geq$ 0.7 were pooled with the first batch. This was concentrated to about 1.5 mL by ultrafiltration as described above.

Gel Permeation Chromatography. The enzyme concentrate was then

loaded onto a Sephacryl S-200 (Pharmacia) gel chromatographic column (80 cm by 1.6 cm) pre-equilibrated with 0.1 M phosphate buffer at pH 7.0. It was eluted with the same buffer at a flow rate of 1 mL/min. Fractions of 5.0 mL were collected and those with $Rz \geq 0.8$ were pooled. These fractions were dialyzed extensively against deionized water overnight.

Activity and Protein Assays. Peroxidase activity was assayed spectrophotometrically by measuring the increase in absorbance at 470 nm upon oxidation of guaiacol (o-methoxyphenol) (28). The assay solution was freshly prepared for each use and stored in a capped dark glass bottle at room temperature. Typically, the assay solution was prepared by mixing 26.90 mL distilled water; 3.0 mL of 0.1 M phosphate buffer pH 7.0; 0.10 mL of 0.1 M H_2O_2 (BDH Chemicals); and 0.048 mL guaiacol (Sigma). Ten microliters of the enzyme preparation was added to a 1-cm path length cuvette containing 3 mL of the assay solution, mixed and the initial change in absorbance at 470 nm measured. If the reaction product is assumed to be only tetraguaiacol which has an extinction coefficient of 26.6 mM^{-1} cm^{-1} then a single unit of enzyme activity is capable of catalyzing the conversion of 0.45 micromoles of substrate per minute.

Protein assays were carried out by the spectrophotometric Bio-Rad protein assay method which uses the Coomassie Brilliant Blue G-250 protein dye.

Molecular Weight Determination. A sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on the enzyme to determine its molecular weight. The gel was run on a Mini-PROTEAN II dual slab cell from Biorad. Twenty five microliters of the sample (0.5 μg protein/μL) was mixed with 25 μL buffer (10 μM Tris-HCl + 1μM EDTA, pH 8.0); 40 μL of 12.5% SDS; 40μL of 25% ß-mercaptoethanol and heated at 100 ^{O}C for 5 minutes. Ten microliters of 0.1% bromophenol blue was added as the tracking dye. The gel was run at a constant current of 18.0 milliamps for 90 minutes. The staining solution used was 0.1% Coomassie blue in 10% acetic acid and the destaining solution was 30% methanol in 10% acetic acid. The gel was dried according to a published procedure (29). The following protein standards were used: phosphorylase b (94 kDa); albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa); and alpha-lactalbumin (14.4 kDa).

The molecular weight was also determined following elution from

Sephacryl S-200 using an 80 cm by 1.6 cm column. The column was calibrated with three proteins of known molecular weight: chymotrypsinogen (25 kDa); ovalbumin (43 kDa); bovine serum albumin (67 kDa).

Spectral and Cyanide-Binding Properties. The optical spectra of the purified enzyme and its cyanide-bound adduct was recorded using a Cary 219 spectrophotometer equipped with a 1-cm path length cuvette. The number of binding sites was determined by titrating 2.5 μM of the enzyme with cyanide and following the change in absorbance at 430 nm.

2.3. RESULTS AND DISCUSSION

The purification scheme and the typical values obtained for the activity and protein assays for the main steps in the enzyme isolation procedure is presented in Table 2.1. The Reinheitzahl (Rz) value which is A_{430}/A_{280nm} is a very convenient criterion for purity. The value reported here is the same as that reported by Agner for crystalline myeloperoxidase from canine pus (2). Moreover, a 105-fold purification was achieved in four major steps in the procedure. Figure 2.1 shows a photograph of the ion exchange chromatographic column. Several protein impurities elute before the green fraction containing the enzyme. The

Table 2.1. Typical values obtained for the activity and protein assays for the main steps in the enzyme purification procedure.

Step in Procedure	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Rz	% Recovery	Purification Grade (Folds)
Acid Extraction	2450	8930	3.6	< 0.01	100	1.0
Batch Adsorption	196	8745	45	0.023	96	13
Ion Exchange	19	3002	158	0.4-0.7	66	44
Gel Permeation	5	1660	378	0.82	19	105

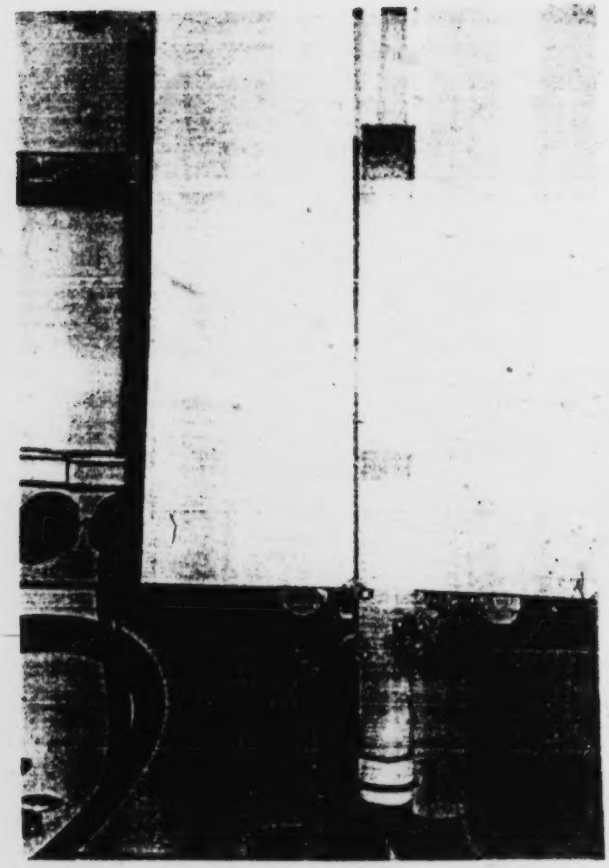

Figure 2.1. Loading the crude enzyme extract to the ion exchange chromatographic column. The dark band on the top of the column contains the myeloperoxidase fraction. Most of the protein impurities (e.g. middle band) are eluted first.

pinkish middle band does not contain any peroxidase activity as verified by the guaiacol assay. It is probably acid phosphatase which is known to be also present in considerable quantities in beef spleen (30). The top green band elutes at an ionic strength of 0.45 M (Fig. 2.2) suggesting that the protein is highly basic. This is consistent with a previously reported isoelectric point for myeloperoxidase (pI=10) (31).

The purity as well as the high yield of the enzyme from beef spleen makes the latter a very practical source of the enzyme. The spleen apparently does a very good job of concentrating the enzyme in the monocytes which are present in significant amounts in this organ (32). Moreover, beef spleen is easily obtained from the local abattoir in large quantities and the enzyme purification procedure is simple.

The molecular weight of the enzyme preparation was estimated using SDS-PAGE and gel permeation chromatography. Figure 2.3 shows a plot of log molecular weight against relative mobility (R_f). Two bands were observed: the major band has a MW of 59 kDa while a minor band gave a MW of 15.3 kDa. These values are in agreement with the molecular weights of the big and small subunits previously reported for myeloperoxidase (22). From these results it was not apparent whether

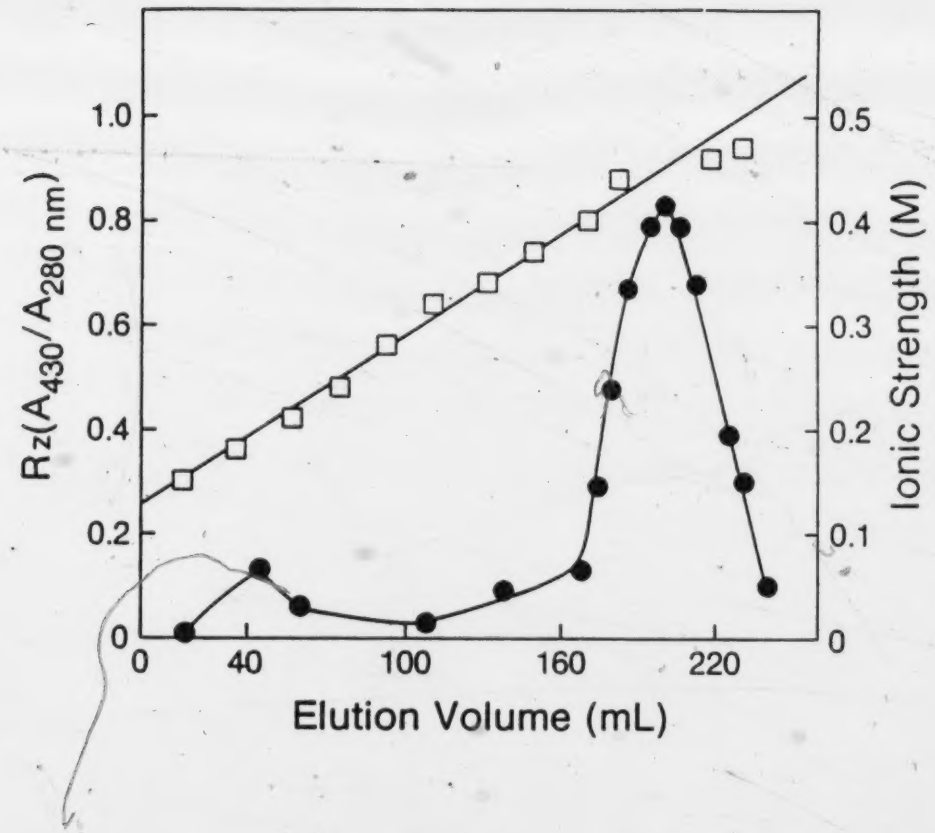

Figure 2.2. Typical elution profile of ion exchange chromatography using CM-Sepharose CL-6B gel.□)Ionic strength gradient (due to phosphate buffer) with 0.1 M as starting concentration and 1.0 M as limiting concentration.●) Purity number (Rz) versus elution volume.

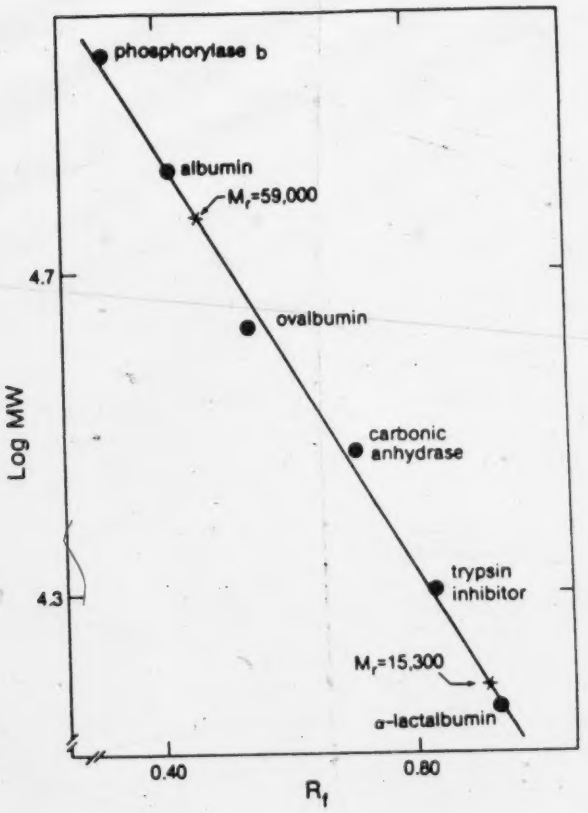

Figure 2.3. Plot of log of molecular weights of protein standards and enzyme sample versus relative mobility (R_f = distance of protein migration/ distance of tracking dye migration) in SDS polyacrylamide gel. The major band of the enzyme preparation has MW= 59 kDa while a minor band exhibits a MW = 15.3 kDa.

the spleen enzyme was a dimer composed of a big and a small subunit or whether it was tetrameric. SDS-PAGE is a destructive method in the sense that it can reduce polymeric enzymes into their subunit components. In fact, it has been reported that the subunit pattern of myeloperoxidase was a function of the way the enzyme was treated in the SDS-PAGE analysis (33). Thus, gel permeation was tried as another method of determining the molecular weight. Figure 2.4 is a linear regression plot of the elution volume ratios of some protein standards versus the log of their molecular weights.The elution volume ratios of these proteins: $(V_e - V_o)/(V_t - V_o)$, where V_o = void volume determined by blue dextran elution; V_t = computed total volume; and V_e = elution volume, is a linear function of the log of molecular weight (34). Using this method the spleen enzyme was found to have a MW of 118 kDa which is quite low. It has been reported that the molecular weight obtained by gel permeation chromatography tends to be considerably smaller than that obtained by other methods, for example, sedimentation equilibrium (3,16). It is conceivable that this is due to an interaction of the enzyme with the gel due to the basic nature of the enzyme. Such interaction would increase the elution volume, V_e, and subsequently decrease the

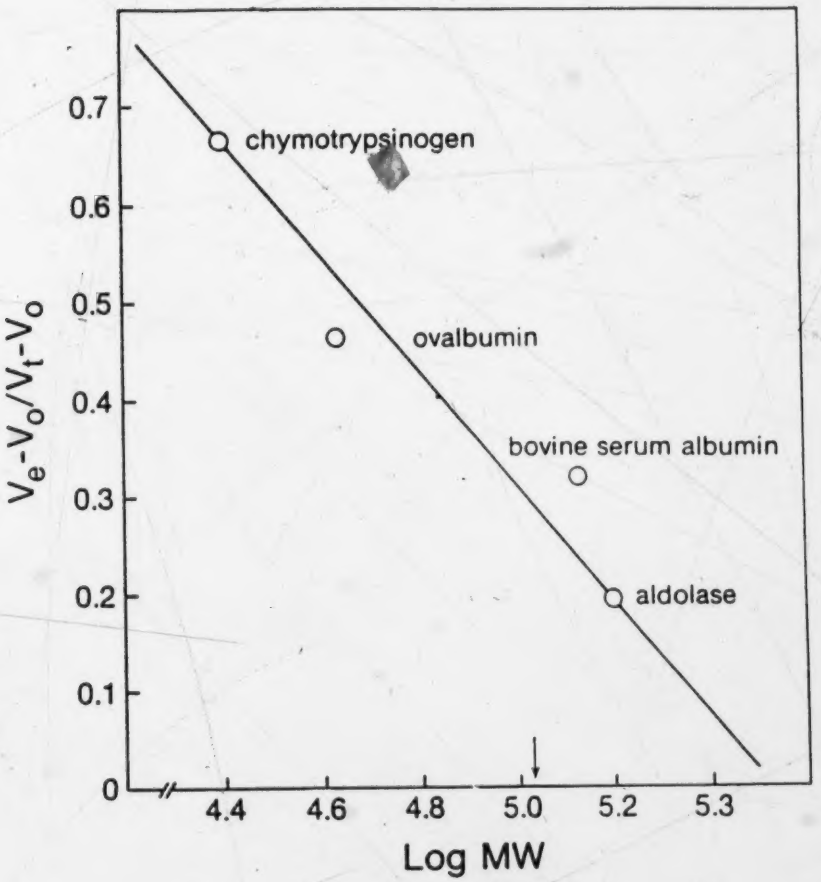

Figure 2.4. MW determination using gel permeation through Sephacryl S-200. The eluting buffer used was 0.1 M phosphate, pH 7.0. Linear regression plot of elution volume ratios (see text for definition) versus log molecular weights of protein standards. Extrapolation gives a MW of 118,000 kDa for the spleen enzyme.

estimated molecular weight.

Figure 2.5 shows the spectra of the native form of the enzyme along with its cyanide adduct. A comparison of spectroscopic properties suggests that the chromophores and the cyanide ligand binding properties of the spleen enzyme and that of canine myeloperoxidase (see Chapter 3) are very similar, if not identical. The peaks at 430 and 570 nm for the native enzyme; and 455 and 635 for the cyanide bound enzyme agree very well with those reported for myeloperoxidase (35). These spectral features are all substantially red shifted compared with the spectra of other hemeproteins suggesting an unusual structure of the heme prosthetic group.

An isosbestic point was observed at 444 nm during the titration of the enzyme with cyanide (Fig. 2.6). Extrapolation of the plot of change in absorbance at 430 nm (to follow disappearance of the native enzyme) versus the amount of cyanide added (Fig. 2.7) suggests that the enzyme has two binding sites. This is consistent with reports that myeloperoxidase has two heme prosthetic groups (3,16) as well as our similar finding for cyanide binding to canine myeloperoxidase. The presence of a single isosbestic point suggests that the binding sites may

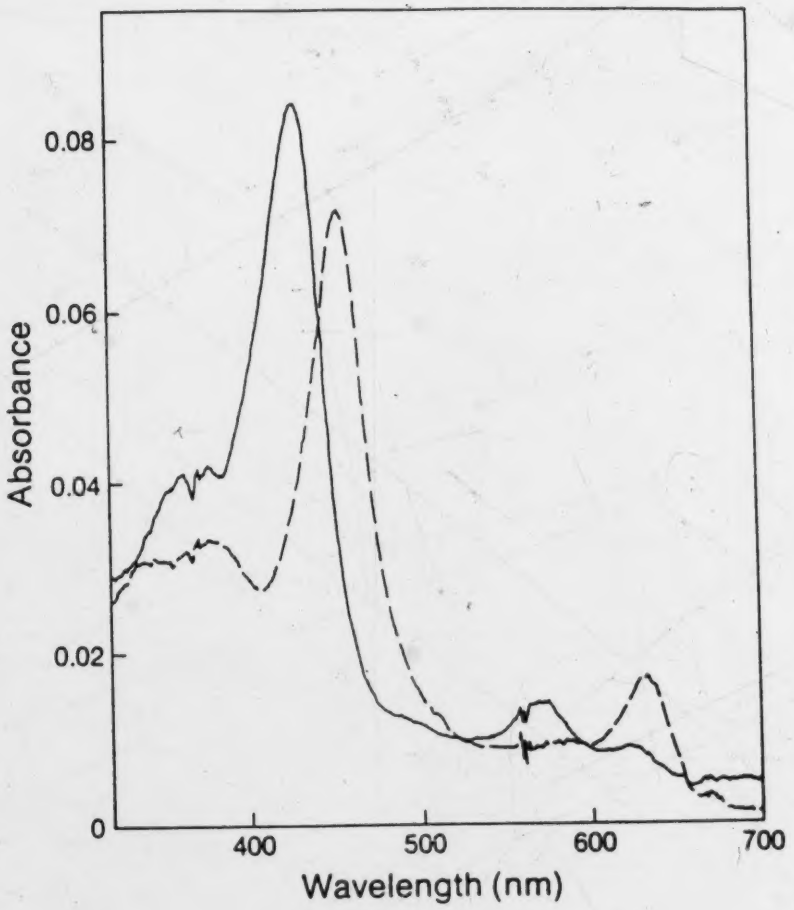

Figure 2.5. Visible spectra of native enzyme and its cyanide adduct. Solid line:Cuvette contains 0.5 μM enzyme in 0.1 M phosphate, pH 7.2. Broken line: 200 μM cyanide was added to the native enzyme.

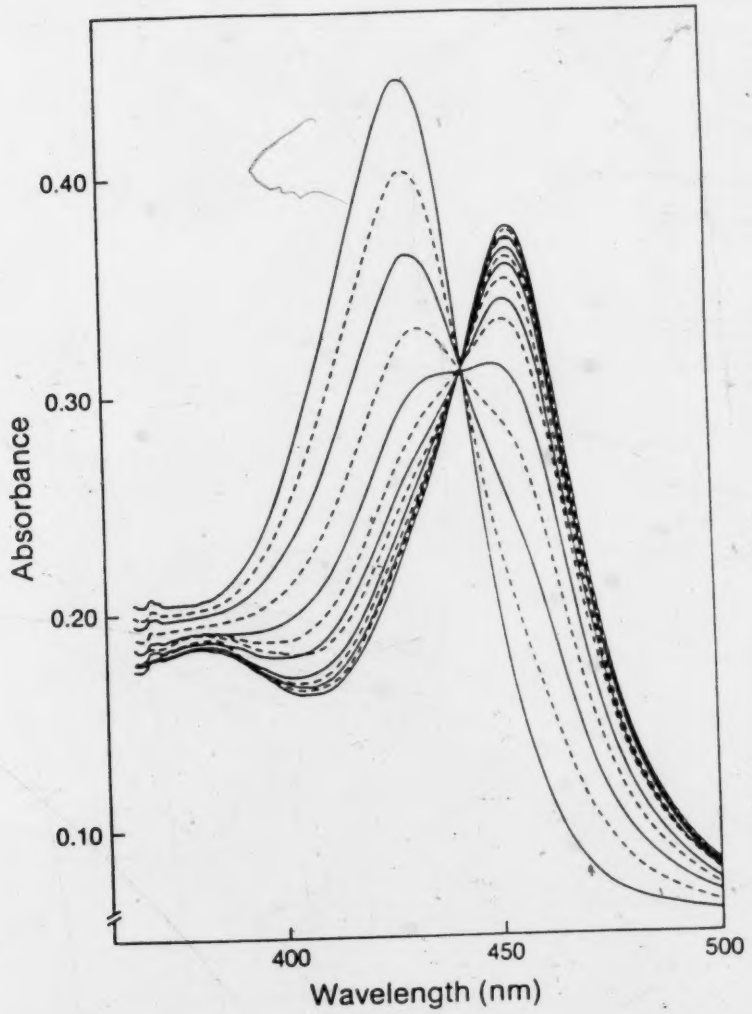

Figure 2.6. Titration of spleen enzyme with cyanide. The reaction cuvette initially contained 2.5 μM enzyme in 0.1 M phosphate, pH 7.4. One μM of KCN was added each time and the spectra recorded. Scans show conversion of native enzyme to its cyanide adduct and an isosbestic point at 444 nm.

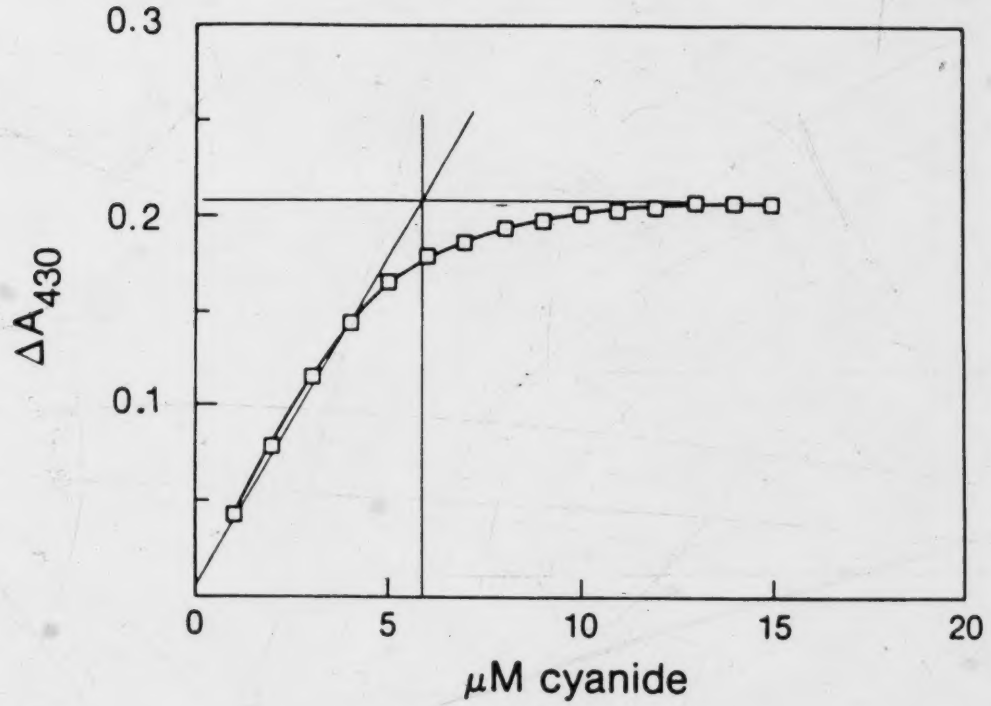

Figure 2.7. Determination of number of binding sites. The change in absorbance at 430 nm corresponding to the disappearance of the native enzyme as it is converted to its cyanide adduct was plotted against μM of cyanide added. Titration data were obtained from Fig. 2.6. Extrapolation of the plot suggests the presence of two binding sites.

be equivalent.

The results of these preliminary characterization studies point to the similarity between myeloperoxidase and the bovine spleen enzyme.

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CHAPTER THREE

CYANIDE BINDING TO CANINE MYELOPEROXIDASE

3.1. INTRODUCTION

Myeloperoxidase has been linked to the body's defense mechanism because of its antimicrobial activity (1,2). Investigations on the structure of myeloperoxidase from various species have shown it to be a two-heme protein with MW in the region of 130,000 to 153,000 kDa. Myeloperoxidase isolated from human neutrophils was observed to consist of two heavy-light dimers which are joined along their long axes by a single disulfide bond between the heavy subunits (3). The canine myeloperoxidase contains two covalently linked carbohydrate-containing large subunits of MW about 57,000 kDa, each of which is apparently associated with a small subunit of MW 10,000 kDa. The large subunits contain the active center hemes (4). More recently Ikeda-Saito (5) isolated a green hemeprotein from bovine spleen and found it to be structurally similar to myeloperoxidase from neutrophil and canine

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A version of this chapter has been published. Marquez, L. A., and Dunford, H. B. (1989) *Biochem. Cell Biol.* **67**, 187-191.

sources, with two heavy subunits (MW 60,000 kDa each with a single prosthetic group per subunit) and two light subunits (MW15,000 kDa each).

No other peroxidase is known to possess a subunit structure as complex as that of myeloperoxidase. It has also been shown that although the two subunits are homogeneous on the basis of their electrophoretic mobilities in dodecyl sulfate, isoelectric focusing revealed a complex pattern of charge heterogeneity in each of the subunits (4). On the other hand, the reductive cleavage of myeloperoxidase gave two species containing both subunit types, all heme prosthetic groups as well as the full specific activity of the intact enzyme (3).

In this study cyanide binding to canine myeloperoxidase was used to investigate further the active site of the enzyme and to gain information as to whether functional inequivalence exists between the two enzyme subunits. The pH dependence of the apparent dissociation rate constant for cyanide complex formation was used in order to help to determine what functional group is involved in the catalysis.

3.2. MATERIALS AND METHODS

Canine myeloperoxidase was prepared as previously described (4).

The enzyme preparation used in this study exhibited an Rz (A_{430}/A_{280}) of 0.80. The myeloperoxidase concentration was determined spectrophotometrically using an extinction coefficient at 430 nm of 178 $mM^{-1}cm^{-1}$ per molecule of enzyme (6).

Potassium cyanide (Fisher Scientific Co., Fair Lawn, N.J.) was reagent grade and used without further purification. The quality of KCN was checked by titration with a standardized $AgNO_3$ solution (7). The cyanide solutions were freshly prepared on each day that the experiments were performed. All experiments were carried out at 25°C and at an ionic strength of 0.11 M, due to the contribution of the buffer. The following buffers were used: citrate (pH 3.11 -4.27); phosphate (pH 5.26 - 8.32) and carbonate (pH 9.05- 9.70). The pH was measured using a Fisher microprobe electrode and Fisher Accumet Model 420 digital pH meter.

Optical absorption spectra were obtained with a Cary 219 recording spectrophotometer. Stopped flow measurements were made using a Photal (formerly Union Giken) rapid reaction analyzer (model RA601) at a wavelength of 430 nm. Pseudo-first-order rate constants were evaluated by an exponential curve-fitting program and the pH profile of the kinetic data was fitted using non-linear least squares analysis.

3.3. RESULTS

Figure 3.1 shows the uv-visible spectra of free canine myeloperoxidase and its cyanide-bound complex. The spectra were recorded from 240 nm to 700 nm. Cyanide binding to myeloperoxidase is accompanied by a large change in the absorption spectrum. The Soret peak at 430 nm is shifted to 454 nm. Other differences in the spectra include the disappearance of the peak at 570 nm for the native enzyme and the appearance of a peak at 634 for the cyanide-bound complex. Isosbestic points were observed at 658, 602, 516 and 445 nm. This agrees with previously reported absorption spectra for canine myeloperoxidase (8).

The spectra of the cyanide-bound enzyme were also recorded at the following pH's : 2.56 (citrate- phosphate buffer); 5.34 (citrate); 8.86 (borate) and 10.94 (bicarbonate). It was found that the spectra were virtually unaffected by pH changes. Moreover, the cyanide-bound complex was found to be very stable even at the extreme pH's. Its spectrum remained the same even after two hours of incubation at pH's 2.56 and 10.94.

Titration experiments were performed to determine the number of binding sites and the dissociation constant. The change in absorbance at

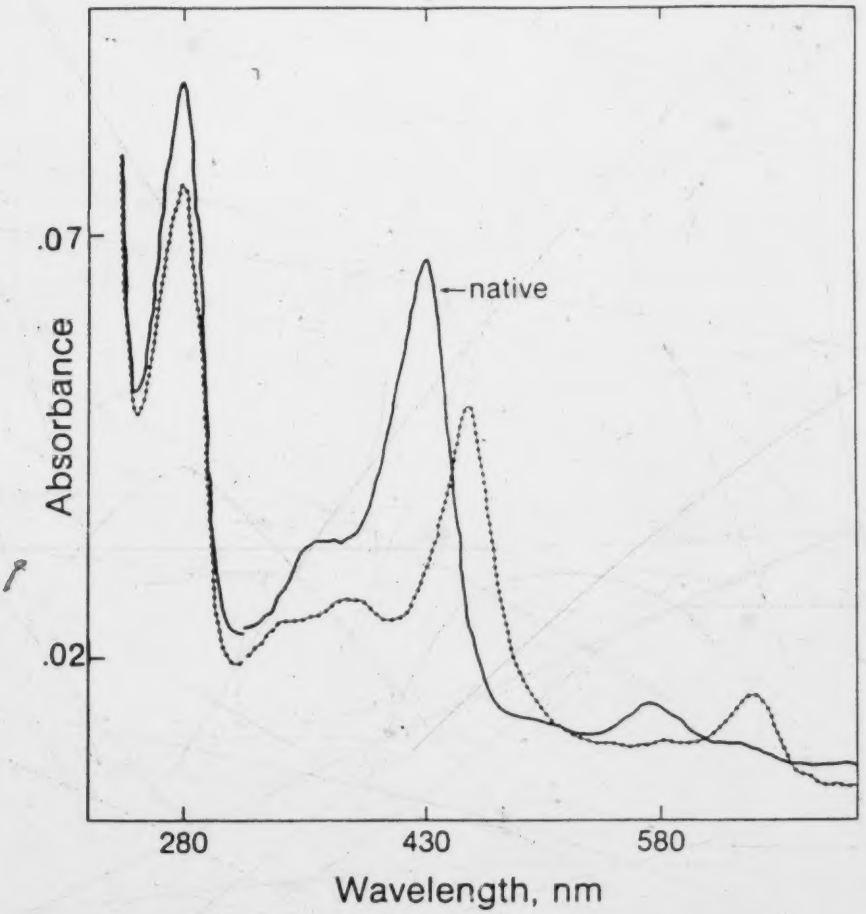


Figure 3.1. UV-visible spectra of native MPO and MPO-cyanide complex. 3.75 μm of enzyme in phosphate buffer (pH 6.96) and upon addition of 0.10 mM KCN.

430 nm was measured over a range of ligand concentrations until addition of ligand caused no further spectral change. Figure 3.2 shows a steady decrease in absorbance at 430 nm as more and more cyanide is added. This peak eventually disappears as the peak at 454 nm (characteristic of the cyanide-bound complex) appears. An isosbestic point at 444 nm is also observed. Both visible and magnetic circular dichroism spectroscopies have been used by other workers and it was reported that there was no single set of isosbestic points during cyanide binding to human leucocyte myeloperoxidase (9). They claimed that there are at least two cyanide binding processes exhibiting different ligand affinity and spectral changes. The data in this work show that canine myeloperoxidase exhibits a different behavior.

To determine the number of binding sites, a plot was made of the absorbance change at 430 nm against total concentration of cyanide added. Extrapolation of the graph (Figure 3.3) shows that for every 2.01 μM of myeloperoxidase approximately 3.9 μM of cyanide is required for full binding. This suggests two binding sites per myeloperoxidase molecule. This is consistent with previous reports that various myeloperoxidases contain two heme prosthetic groups (4, 10,11).

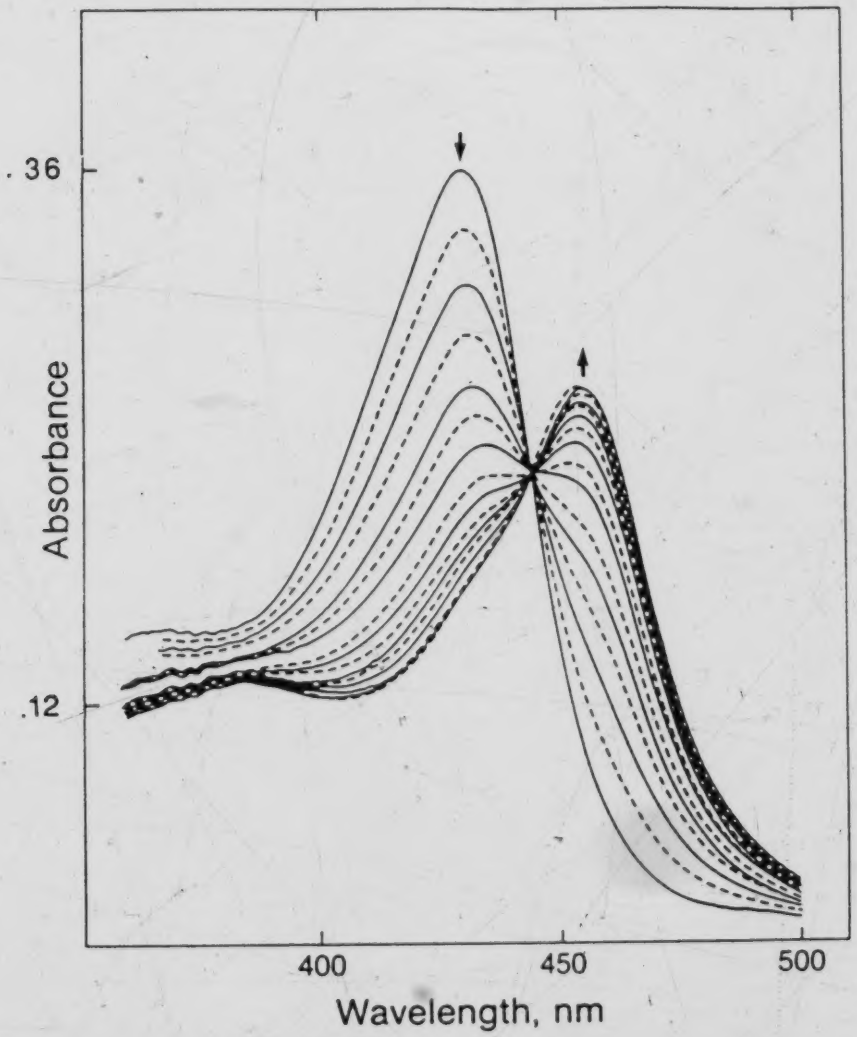


Figure 3.2. Titration of MPO with cyanide. Blank cuvette contained phosphate buffer while the sample cuvette contained 2.01 μM MPO in same buffer (pH 7.04 ; ionic strength = 0.1 M). Microliters of 0.10 mM KCN were successively added to both cuvettes and the spectra scanned from 360-500 nm. Arrows show direction of change of absorbance.

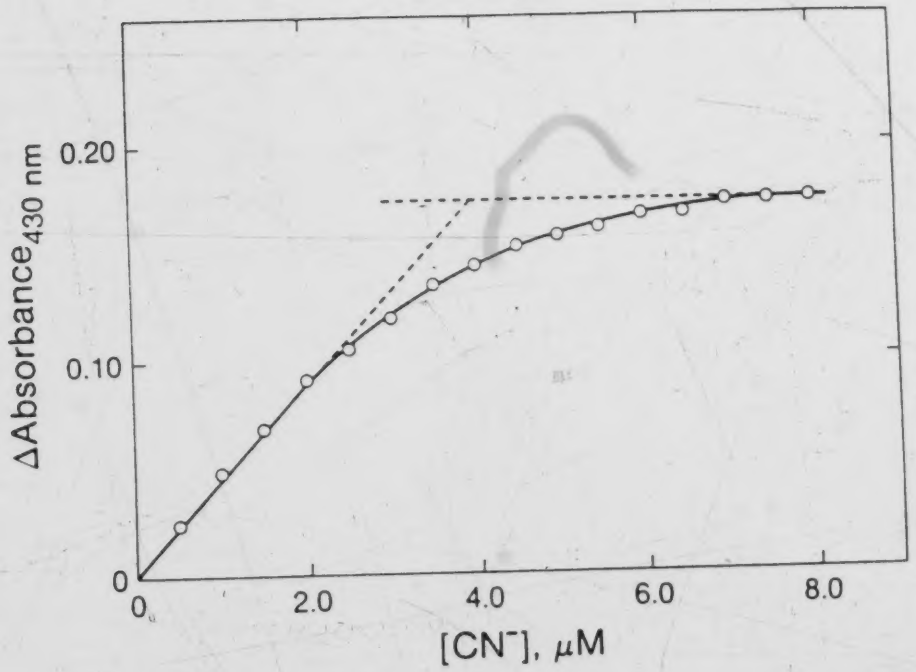


Figure 3.3. Number of binding sites of MPO. Same conditions as in Fig. 3.2. Absorbance changes were followed at 430 nm. Extrapolation of the graph shows that 2.01 μM MPO binds 3.9 μM of cyanide suggesting two binding sites per enzyme molecule.

The difference in absorbance with increasing concentrations of cyanide was used to determine the dissociation constant for cyanide binding, K_{CN}, by means of a Scatchard plot (Figure 3.4). K_{CN} was found to be $5.3 \pm 0.1 \times 10^{-7}$ M; thus, cyanide binds very tightly to the enzyme. Whereas a straight line Scatchard plot does not unequivocally prove that the heme groups have the same cyanide binding affinity and act independently (12), the fact that myeloperoxidase has been cleaved to two identical dimers with similar catalytic activities (3) suggests that the binding sites in myeloperoxidase may be identical. A comparative plot was made using the Bjerrum (semi-log) plot (Figure 3.5) and a K_{CN} value of 7.0×10^{-7} M was obtained. These values are comparable with values previously obtained: 4.30×10^{-7} M for human blood myeloperoxidase (13); and 6.0×10^{-7} M for bovine spleen myeloperoxidase (14). Like the above workers, the enzyme is treated here as though it is composed of two independent subunits, since this is in accord with the experimental results. Thus, within this context, the units of M are correct for the dissociation constant.

Pseudo-first order kinetics of cyanide binding to myeloperoxidase was investigated. For stopped flow measurements one reservoir

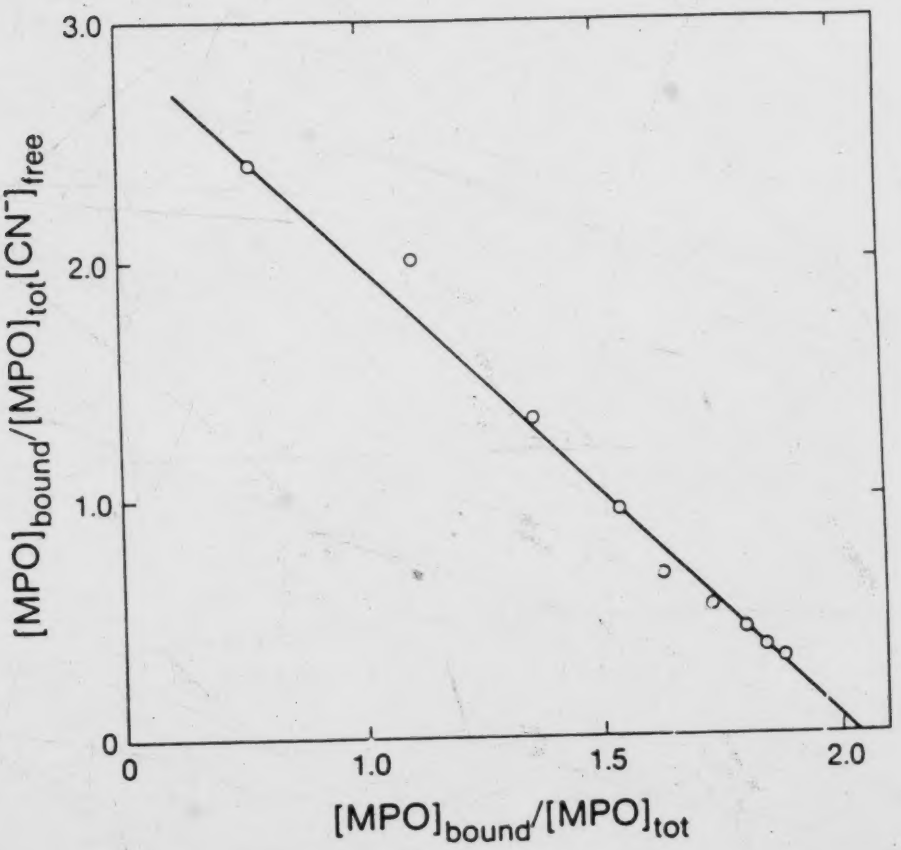


Figure 3.4. Scatchard plot constructed from absorbance changes at 430 nm with increasing concentrations of cyanide (in μM). 2.19 μM enzyme in phosphate buffer (pH 7.04 ; ionic strength = 0.1 M) at 25 ^{o}C. The plot gives a dissociation constant of 5.3 ± 0.1 X 10^{-7} M.

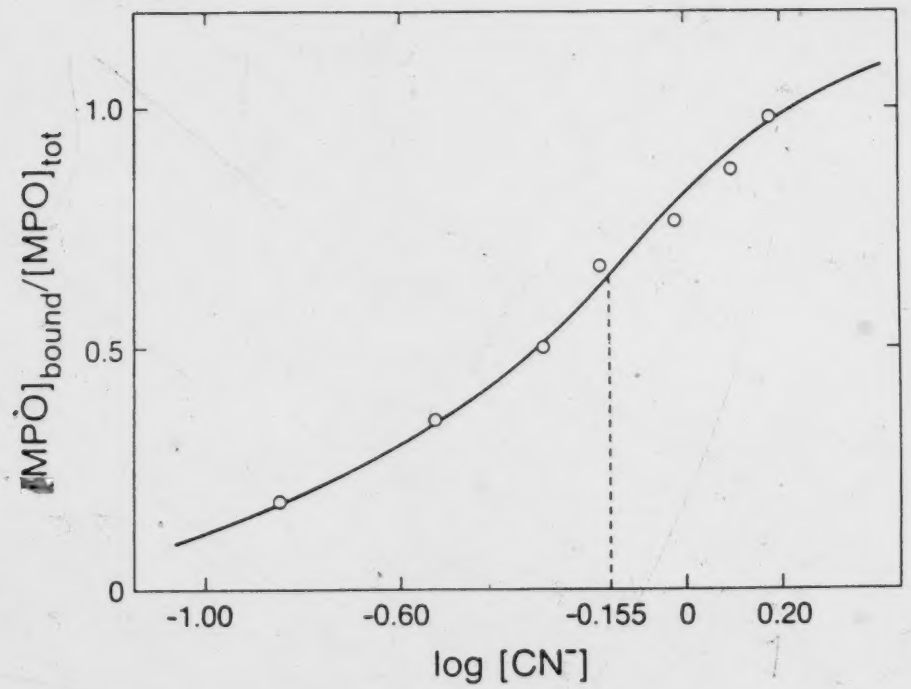


Figure 3.5. Bjerrum (semilog) plot. Same conditions as in Fig. 3.4. The cyanide concentrations are in μM. The dissociation constant obtained from the inflection point was 7.0 X 10^{-7} M.

contained 2.0 μM myeloperoxidase in 0.22 M ionic strength buffer and the other reservoir at least a 20-fold excess of cyanide in aqueous solution.

In the initial experiments, the reaction was followed at 430 nm (to monitor the rate of disappearance of native enzyme) and at 454 nm and 458 nm (15) (to monitor the rate of formation of the cyanide-bound complex). The difference in the wavelength used did not cause any significant discrepancy in the observed rate constants: at 430 nm: k_{obs} = $3.9 \pm 0.2 \times 10^2 s^{-1}$; at 454 nm: k_{obs}= $4.2 \pm 0.1 \times 10^2 s^{-1}$; and at 458 nm: k_{obs}= $3.5 \pm 0.2 \times 10^2 s^{-1}$. For subsequent kinetic runs, 430 nm was used to measure the rate constants because it gives a greater change in absorbance upon cyanide binding (see Figure 3.1).

The observed rate constant increases linearly with the concentration of cyanide (Figure 3.6) demonstrating second-order kinetics represented by equation 3.1. (See appendix for the derivation).

[3.1] $$k_{obs} = k_{1app} (CN^-) + k_{-1app}$$

where k_{1app} is the apparent second order association rate constant and k_{-1app} is the apparent dissociation rate constant. No rate saturation was observed over the range of 20-120 μM cyanide. The k_{-1app} cannot be

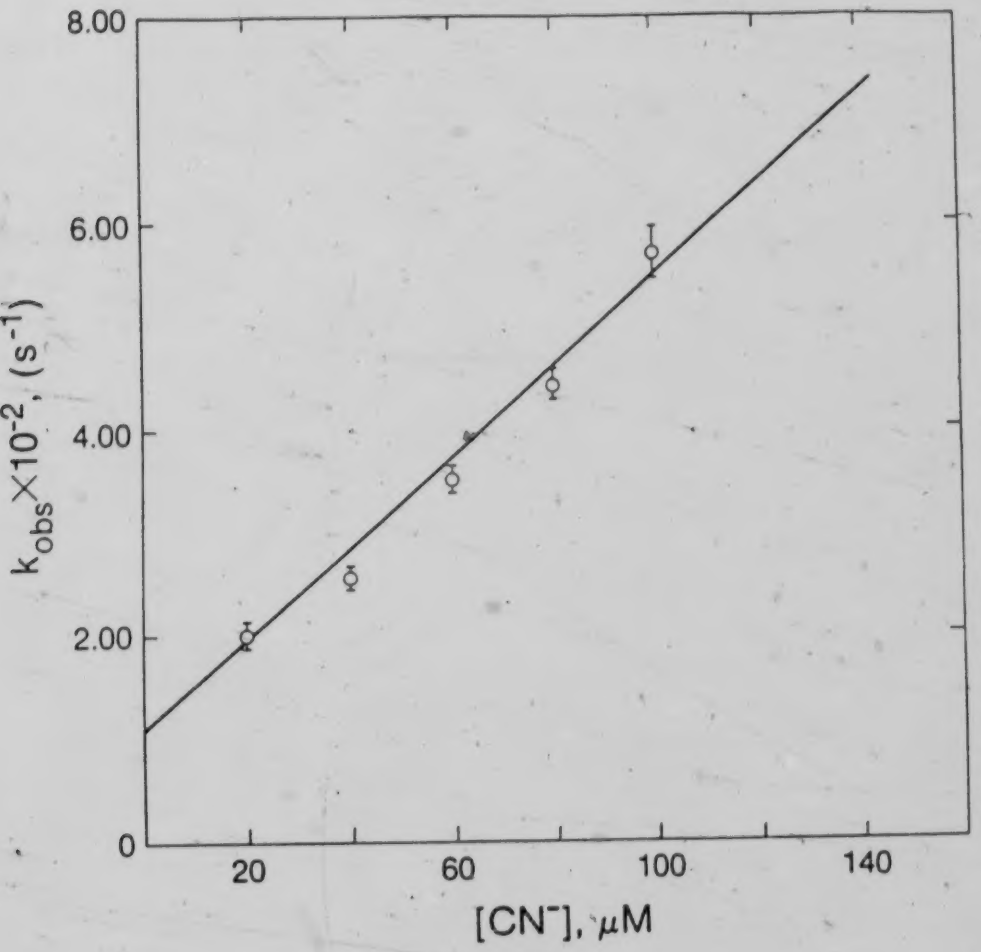


Figure 3.6. Pseudo-first order rate constants of cyanide binding to MPO plotted as a function of total cyanide concentration. 1.00 μM MPO in phosphate buffer (pH 7.04 ; ionic strength = 0.11 M) at 25 ^{o}C. The error bars show the standard deviation calculated from 6-8 measurements of k_{obs} at each cyanide concentration. The slope gives a value of 4.1 ± 0.5 x 10^6 M^{-1} s^{-1} for k_{1app}.

accurately determined from the intercept.

Pseudo-first order conditions were again used to determine the dependence of k_{1app} on pH. Figure 3.7 shows that the apparent second order rate constant decreased at extremes of pH and was at its maximum from pH 5.5 to 7.0. The lack of an effect of pH on cyanide dissociation rate constants around neutral pH has been reported for horseradish peroxidase, myeloperoxidase and chloroperoxidase (13,16,17).

Using non-linear least squares analysis, Eq. [3.2] was used to fit the data points :

[3.2] $$k_{1app} = k_1 / (1 + (H^+)/K_1)(1 + K_a/(H^+))$$

and the parameters found were: $k_1 = 4.3 \pm 0.3 \times 10^6\ M^{-1}s^{-1}$, $K_1 = 1.5 \pm 0.4 \times 10^{-4}$, $pK_1 = 3.8 \pm 0.1$; $K_a = 1.1 \pm 0.2 \times 10^{-9}$, $pK_a = 9.0 \pm 0.1$. The pK_a of hydrocyanic acid is 9.31 at 25^{0}C (CRC Handbook of Chemistry and Physics) and thus, the K_a calculated is due to the dissociation of HCN. pK_1 indicates the strength of an acid-base group in the active site of the enzyme that influences the binding of cyanide to myeloperoxidase. These values are comparable with those obtained for spleen myeloperoxidase : $k_1 = 2.46 \pm 0.03 \times 10^6\ M^{-1}s^{-1}$ and a $pK_1 = 3.96 \pm 0.03$ at 20 ^{0}C (15) ; and

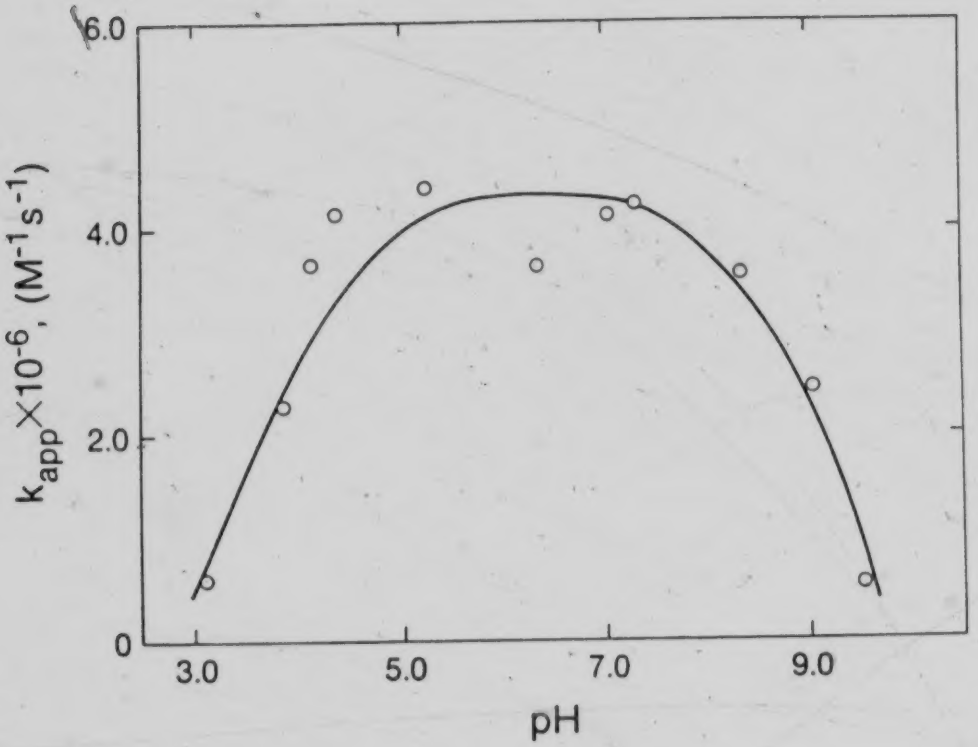
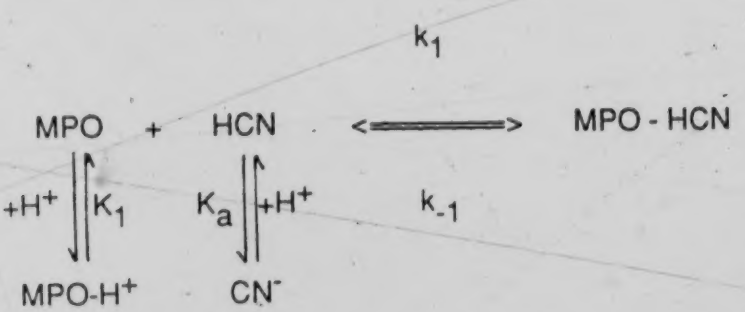


Figure 3.7. pH dependence of the apparent association rate constant of cyanide binding. Same conditions as Fig. 3.6 except that pH was varied from 3.11 to 9.52. The curve was computed on the basis of best-fit parameters obtained from a non-linear least squares analysis of the data to Equation [3.2].

for human granulocyte myeloperoxidase: $k_1 = 1.30 \pm 0.05 \times 10^6\ M^{-1}s^{-1}$ and $pK_1 = 4.00 \pm 0.05$ (13).

The scheme which appears to account for these parameters is shown in 3.3.

[3.3]



3.4. DISCUSSION

Myeloperoxidase has a relatively faster association rate for cyanide complex formation than other peroxidases as shown in Table 3.1. Unlike the other peroxidases which possess a porphyrin prosthetic group, myeloperoxidase appears to have a chlorin prosthetic group (8,21). Where a metalloporphyrin has planar pyrrole rings, a metallochlorin shows loss of planarity; this results in a dramatic reduction of aromaticity relative to the porphyrins. This difference has been invoked to explain the increased ligand affinity of myeloperoxidase relative to the other

TABLE 3.1. Cyanide association rate constants of some peroxidases

peroxidase	rate constant ($M^{-1} s^{-1}$)	reference
canine myeloperoxidase	$4.3 \pm 0.3 \times 10^6$	(this work)
leucocyte myeloperoxidase	$1.3 \pm 0.1 \times 10^6$	(13)
horseradish peroxidase	$1.1 \pm 0.1 \times 10^5$	(18)
cytochrome c peroxidase	$6.5 \pm 0.2 \times 10^4$	(19)
chloroperoxidase	$5.2 \pm 0.5 \times 10^4$	(20)

peroxidases (22).

Spectral evidence suggests that the subunits of myeloperoxidase exhibit similar ligand affinity.

The pH profile of the apparent second order rate constant for cyanide binding shows that an acid/base group with $pK_a = 3.8 \pm 0.1$ is present on the enzyme which when protonated prevents binding of cyanide. In other words, the protonated form of cyanide binds to the unprotonated form of the enzyme, which is in agreement with related studies outlined below.

Equilibrium binding studies on ferric chloroperoxidase shows that weakly acidic ligands like cyanide bind to the enzyme in their protonated form followed by deprotonation upon ligation to the heme iron (17). For the reaction between horseradish peroxidase and hydrogen peroxide, the scheme that fits the kinetic data involves the reaction of the neutral substrate (23). A kinetically equivalent mechanism wherein unprotonated HO_2^- reacts with the protonated form of the enzyme was eliminated on the basis that it leads to a rate constant far in excess of the diffusion-controlled limit. Studies on azide binding to chloroperoxidase showed that the most plausible mechanism for the reaction is one in which hydrazoic

acid binds to the enzyme. It was proposed that the proton from hydrazoic acid is transferred to a basic distal group which facilitates azide binding to the heme iron (24).

The pH dependence of the dissociation rate constant has been used to show a linkage between ligand binding and ionization or proton uptake of a group in the enzyme. The decrease in the apparent rate constant in the alkaline region is readily attributed to the ionization of hydrocyanic acid. On the other hand, as the pH is lowered from neutral to acidic values, a functional group becomes protonated and the enzyme's affinity for cyanide is reduced. It has been proposed that HCN replaces the water molecule present as the sixth heme ligand via hydrogen bonding to the amino group (13). Evidence for a 6-coordinated high spin configuration with a water molecule as the sixth ligand for the resting state of myeloperoxidase is provided by both resonance Raman spectra and proton magnetic resonance (21,25,26). Thus, myeloperoxidase appears to be different from other peroxidases, such as horseradish peroxidase, which are five-coordinate (27). Figure 3.8 shows the ferric center of the enzyme with a water molecule as the sixth ligand hydrogen bonded to a basic distal group (B). When this group is unprotonated HCN can gain

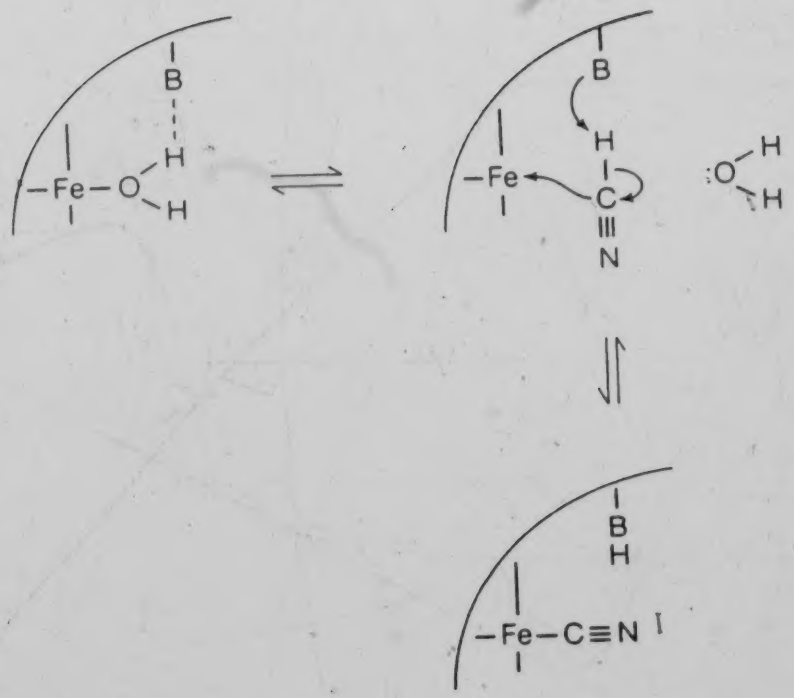


Figure 3.8. Schematic representation of the binding of cyanide to the iron centre of canine myeloperoxidase. B is the basic group; the pK_a of HB is 3.8 ± 0.1.

access to the active center of the heme by transferring its proton to B . The scheme presented here does not address the question of whether the water molecule departs before HCN is bound or whether the water is displaced by HCN. On the basis of available evidence we cannot choose between a dissociative or associative mechanism, although considering the steric hindrance, a dissociative mechanism would be favored for the six-coordinate ferric center.

The imidazole group of a histidine residue located near the ligand binding site has been implicated to control access to the binding site of the enzyme (13,15). It has been further suggested that the distal histidine traps the proton released from HCN upon binding so that it directly participates in the cyanide-binding reaction.

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CHAPTER FOUR

COMPOUNDS I AND II OF BOVINE SPLEEN MYELOPEROXIDASE

4.1. INTRODUCTION

Myeloperoxidase, which catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ions is one of the essential components of the antimicrobial system of leucocytes. The reaction of myeloperoxidase with hydrogen peroxide initially results in the formation of compound I which oxidizes chloride ions to hypochlorous acid. During turnover, compound I is also converted to compound II via extraneous reactions (1,2).

The normal catalytic cycle of peroxidases is shown in Chapter 1. The optical and spectral properties of native peroxidases and the enzyme intermediates have been extensively reviewed (3).

Myeloperoxidase is a tetrameric protein possessing an iron chlorin as prosthetic group (4-7). These factors make it different from other peroxidases which usually are monomeric and contain ferriproto-

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A version of this chapter has been submitted for publication.

porphyrin IX as prosthetic group. However, like the other peroxidases, it forms the normal enzyme intermediates, compounds I and II. In this chapter, the optical spectra of compounds I and II of bovine spleen myeloperoxidase as well as the rates of formation of these intermediates using hydrogen peroxide as substrate are presented.

4.2. MATERIALS AND METHODS

Spleen myeloperoxidase was isolated using the procedure described in Chapter 2. The enzyme preparation used in this study exhibited an Rz value (A_{430}/A_{280nm}) of 0.80 or better. The myeloperoxidase concentration was determined spectrophotometrically using an extinction coefficient at 430 nm of 178 $mM^{-1}cm^{-1}$ per molecule of enzyme (8). Hydrogen peroxide(BDH Chemicals) was standardized by the oxidation of iodide ion to I_3^- using horseradish peroxidase as catalyst (9).

Stopped flow and rapid scan experiments were performed using a Photal (formerly Union Giken) rapid reaction analyzer (Model RA 601) equipped with a 1-cm observation cell thermostated at 25.0 ± 0.5 °C. The rapid scan absorption spectra of compound I are measured by means of a

multichannel photodiode and stored in a digital computer system. The analog replica is obtained on an X-Y recorder. Spectral regions of 96 nm were scanned centered at about 430 nm. 2.0 μM solution of myeloperoxidase in 0.2 M phosphate buffer, pH 7.1 was mixed with 40 μM of H_2O_2 (concentrations after mixing: 1.0 μM myeloperoxidase; 20 μM H_2O_2; ionic strength = 0.1 M). For recording the spectrum of the native enzyme, the peroxide solution was replaced with water.

For stopped flow measurements one driving syringe contained 1.0 μM myeloperoxidase in 0.2 M ionic strength phosphate buffer and the other at least a 10-fold excess of hydrogen peroxide in aqueous solution. The rate of compound I formation was followed at 430 nm. Pseudo-first order rate constants were evaluated by an exponential curve-fitting program.

For compound II, optical absorption spectra were taken using the Cary 219 spectrophotometer. The spectrum of the native enzyme was obtained using 1.0 μM enzyme in 0.1 M phosphate buffer, pH 7.1. Fifty micromolar H_2O_2 was then added and the spectrum recorded.

To determine the ratio of enzyme to H_2O_2 that gives the best

compound II spectrum, 0.5 μM of myeloperoxidase was titrated with H_2O_2. Repetitive scans of the decay of compound II were also taken from 350 to 700 nm to check the stability of this intermediate.

The rate of compound II formation was followed at 455 nm, close to the isosbestic point of compound I and native enzyme and where the maximum change in absorbance occurred upon formation of compound II. Stopped flow measurements were performed with one driving syringe containing 2.0 μM myeloperoxidase in 0.2 M phosphate buffer at pH 7.1 and the other syringe 20-240 μM H_2O_2 in aqueous solution.

4.3. RESULTS AND DISCUSSION

Peroxidase compound I is typically readily distiguished from the native form of the enzyme by its reduced absorbance in the Soret region. A good spectrum of compound I of spleen myeloperoxidase is obtained only when a 20-fold excess of hydrogen peroxide is used. A smaller excess (2-10 times) led to incomplete compound I formation (spectra not shown); whereas a large excess (40-fold) led to the fast formation of compound II which exhibits a Soret maximum at 455 nm (Fig. 4.1).

Figure 4.2 shows the optical spectra obtained after mixing

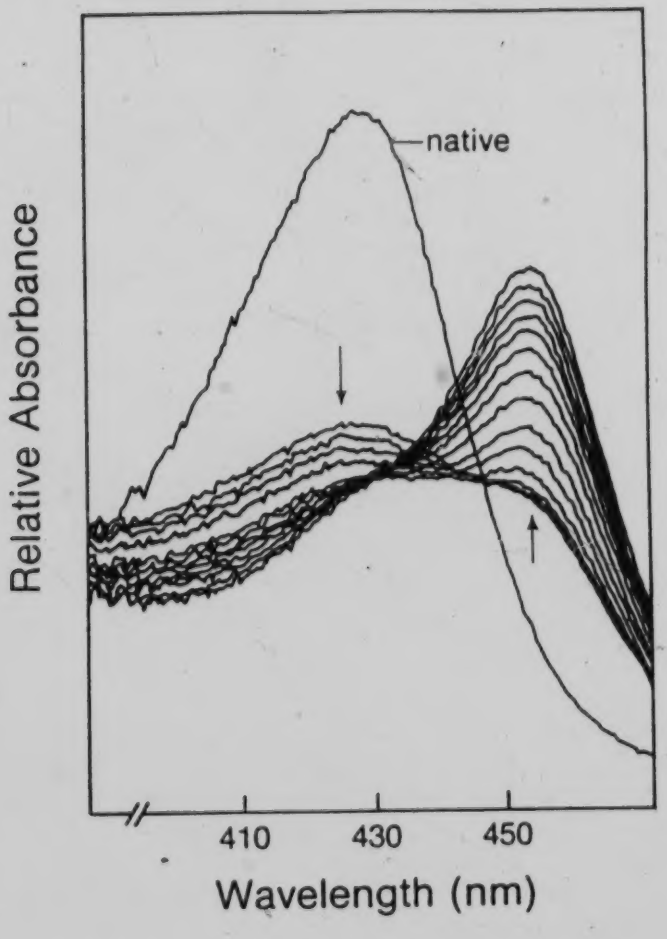

Figure 4.1. Rapid scan spectra of the reaction of spleen myeloperoxidase with H_2O_2. Final concentrations of reactants: 1.0 μM enzyme, 40 μM H_2O_2 in 0.1 M phosphate buffer pH 7.1 at 25 ^{O}C. The first scan was taken 3 ms after the flow stopped and the subsequent scans in 1 ms intervals. The arrows show direction of absorbance changes with time.

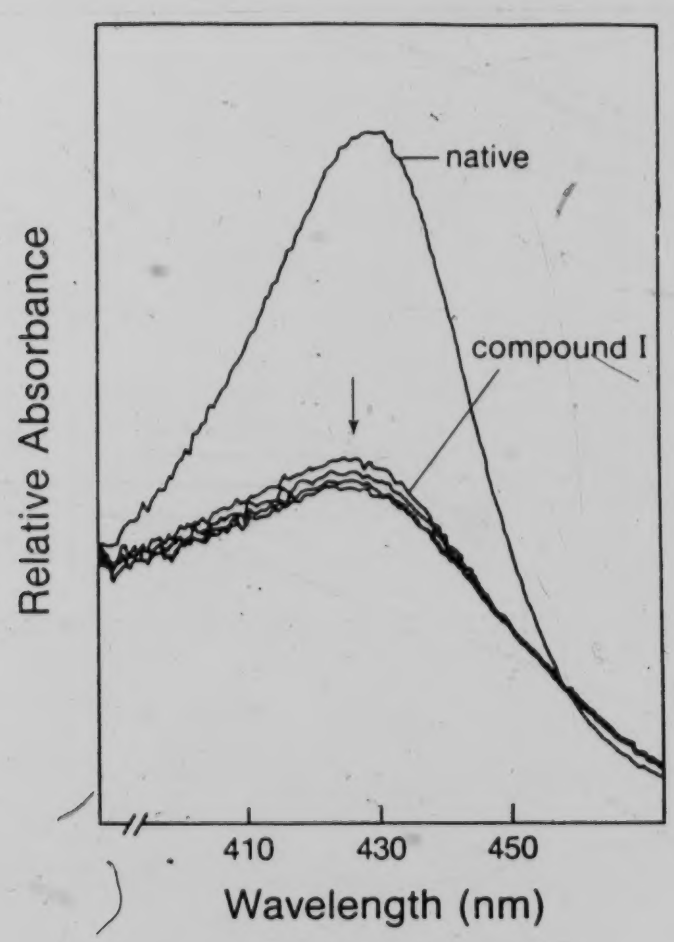

Figure 4.2. Rapid scan spectra of native spleen myeloperoxidase and after adding a 20-fold excess of H_2O_2. The spectra were taken 4-7 ms after the flow stopped. Final enzyme concentration: 1.0 μM in 0.1 M phosphate buffer, pH 7.1. Arrow shows direction of change of absorbance.

myeloperoxidase with a 20-fold excess of hydrogen peroxide 4-7 ms after the flow had stopped. The spectra obtained for the formation of compound I from ferric myeloperoxidase exhibited an isosbestic point at 458 nm and involved broadening and a decrease in the molar absorptivity at 430 nm of about 50%. This broadening and diminution in the Soret absorption has been observed accompanying the formation of compound I of other peroxidases (10).

The preparation of a rather stable compound I has been previously reported although not unequivocally confirmed. The workers added an equimolar amount of H_2O_2 to native enzyme and the spectrum obtained showed just a slight absorbance decrease at 430 nm (2). In this present work a good spectra of compound I was obtained as indicated by the broadening and diminution of the Soret absorption (Fig. 4.2). This preparation also displayed the characteristic instability of the compound I intermediate of other peroxidases. In the presence of a 20-fold excess of H_2O_2 compound I decays to compound II just 8 ms after mixing. Moreover, an excess of H_2O_2 had to be added in order to obtain a good compound I spectrum. Previous workers determined that a 40-fold excess

of H_2O_2 is required for full formation of compound I of canine myeloperoxidase (11). Why would such a large excess of H_2O_2 be required to form compound I? It was thought at first that the enzyme preparation had catalase impurities. However, this explanation was excluded since catalase is an acidic enzyme (pI = 5.5) and therefore it would have been removed during the two cation-exchange chromatographic steps in the isolation procedure. Agner (12) had suggested the cooperation of the two iron sites in the catalase-like activity of the enzyme. Iwamoto and co-workers (13) showed that the abnormal stoichiometry of myeloperoxidase's reaction with H_2O_2 is due to the enzyme's true catalase activity. Compound I reacted with H_2O_2 and returned to the ferric state with concomittant evolution of an O_2 molecule [4.1].

[4.1] $$\text{MPO-Compound I} + H_2O_2 \longrightarrow \text{native MPO} + O_2 + H_2O$$

In this present work with bovine spleen myeloperoxidase it was found that a 20-fold excess of hydrogen peroxide was sufficient to provide a good spectrum of compound I which is also slightly more stable than the canine enzyme intermediate previously reported (11). A larger

excess of hydrogen peroxide enhances the spontaneous conversion of compound I to compound II.

Pseudo-first order kinetics of the reaction were followed at 430 nm. This is the isosbestic point of compounds I and II and at this wavelength the maximum change of absorbance occurred upon formation of compound I (see Fig. 4.1).

The inset of Fig. 4.3 shows a typical trace of compound I formation displaying a single exponential character. The k_{obs} values obtained were found to increase linearly with the concentration of H_2O_2 (Fig. 4.3). The apparent second order rate constant is $1.5 \pm 0.1 \times 10^7\ M^{-1}s^{-1}$. This is comparable to values previously reported: $1.0 \pm 0.2 \times 10^7\ M^{-1}s^{-1}$ for rat chloroma myeloperoxidase (14); $2.0 \times 10^7\ M^{-1}s^{-1}$ for canine pus myeloperoxidase (15); and $2.3 \times 10^7\ M^{-1}s^{-1}$ for human leucocyte myeloperoxidase(16). This is also the same order of magnitude as compound I formation of horseradish peroxidase: $1.8 \pm 0.4 \times 10^7\ M^{-1}s^{-1}$ (17).

An interesting feature of the plot of k_{obs} versus $[H_2O_2]$ is a definite intercept which represents a reverse rate constant. This suggests that the reaction may involve a reversible precursor complex

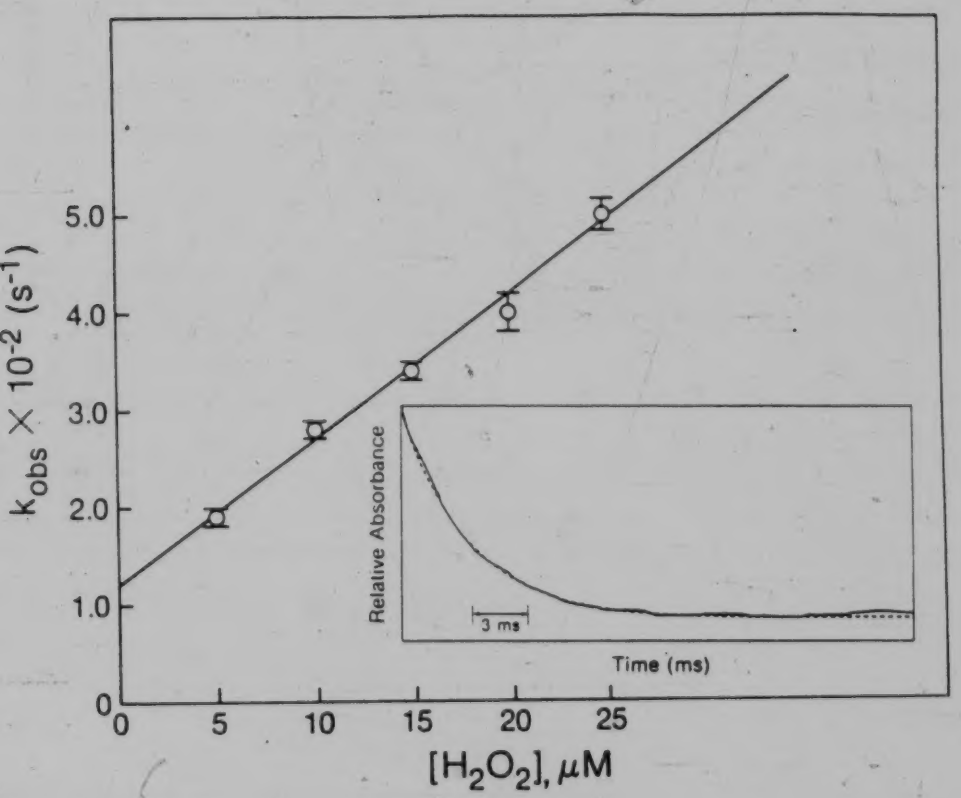

Figure 4.3. Pseudo-first order rate constants for compound I formation plotted against $[H_2O_2]$. Inset shows a typical trace of the reaction followed at 430 nm. Final enzyme concentration: 0.5 μM enzyme in 0.1 M phosphate buffer, pH 7.1. The apparent second order rate constant obtained was $1.5 \pm 0.1 \times 10^7$ $M^{-1}s^{-1}$.

formation before enzyme oxidation. This mechanism can also provide a possible additional explanation for the large excess of H_2O_2 required for compound I formation.

Figure 4.4 shows the Soret-visible spectra of native bovine spleen myeloperoxidase and the compound II intermediate. The peaks at 430 and 570 nm were shifted to 455 and 628 nm. It requires 17-20 fold excess of H_2O_2 for complete formation of compound II at pH 7.1 (Fig. 4.5). The maximum conversion of native enzyme to compound II occurred at the most basic pH of the experiments, 9.8. Figure 4.6 shows the spontaneous decay of compound II to native enzyme isosbestic at 442, 586 and 670 nm. The stability of compound II at higher pH had been reported earlier (18). The peak values for bovine spleen myeloperoxidase compound II agree with those previously reported for human myeloperoxidase compound II (19-20).

Figure 4.7 shows typical experimental and curve-fit traces of the reaction at two different concentrations of hydrogen peroxide. At a lower excess of H_2O_2 (10-fold) the curve fit was not very good but it improved as the concentration is increased to 120-fold (compare A and B). When the concentration of H_2O_2 is more than 40 times that of the

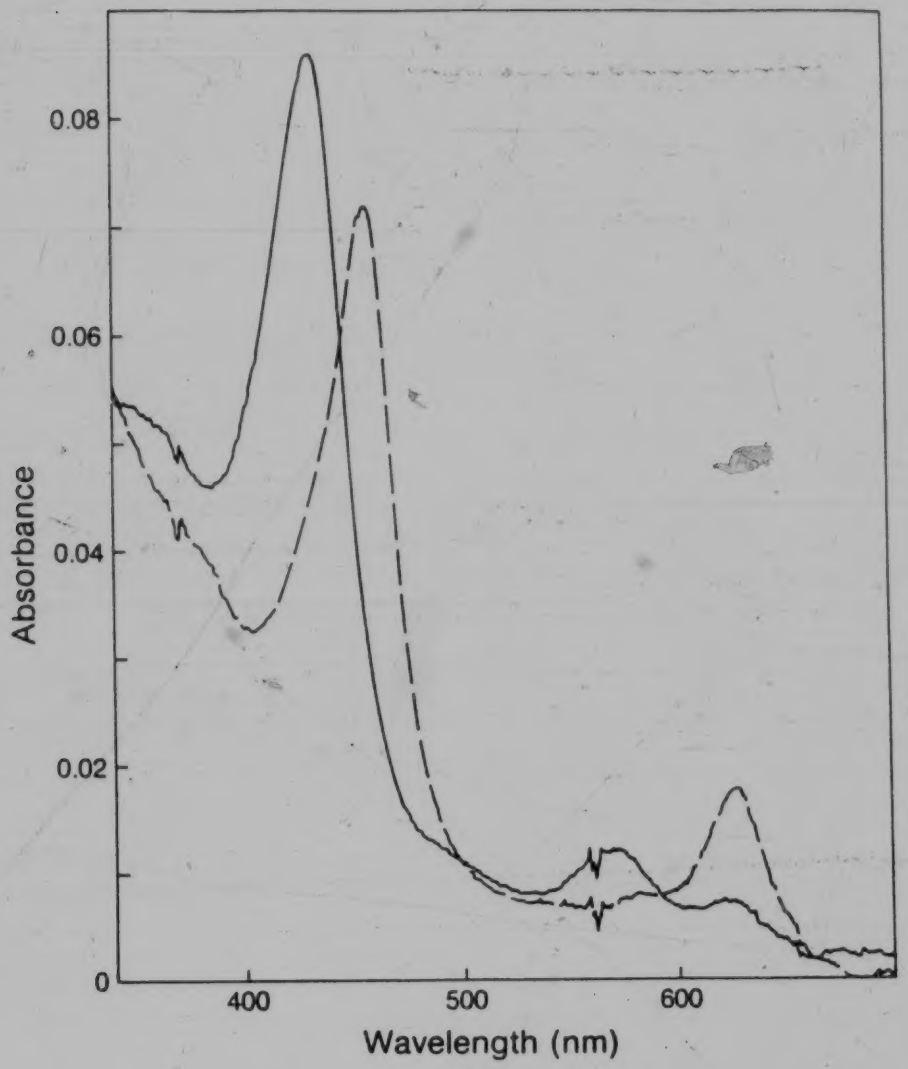

Figure 4.4. Soret/visible spectra of native spleen myeloperoxidase and compound II. Solid line is for 1.0 μM of the enzyme; broken line is after addition of 50 μM H_2O_2.

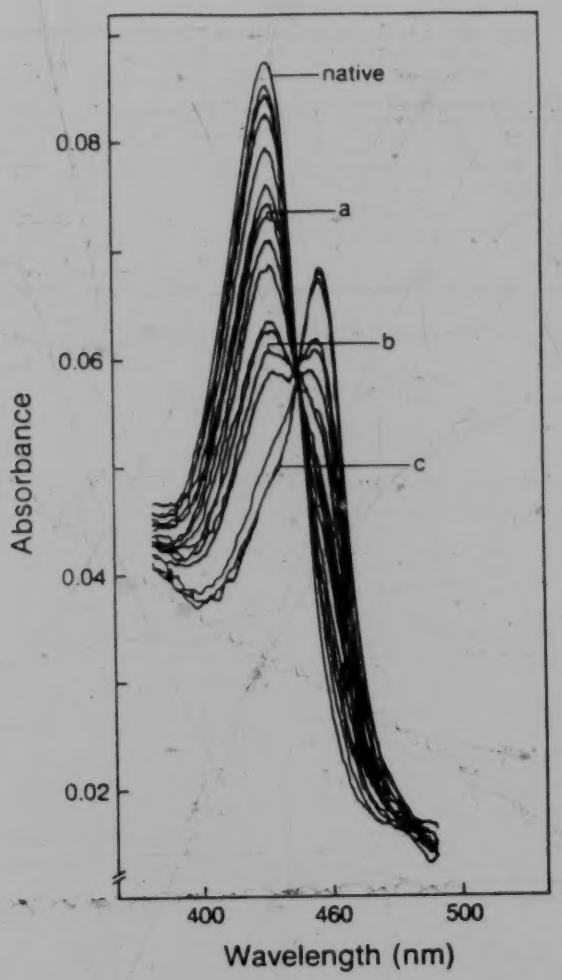

Figure 4.5. Titration of 0.5 μM spleen myeloperoxidase with H_2O_2 at pH 7.1. a) 5-fold excess; b) 10-fold; c) 20-fold. Thus, it requires about 20 fold excess of H_2O_2 for complete formation of compound II.

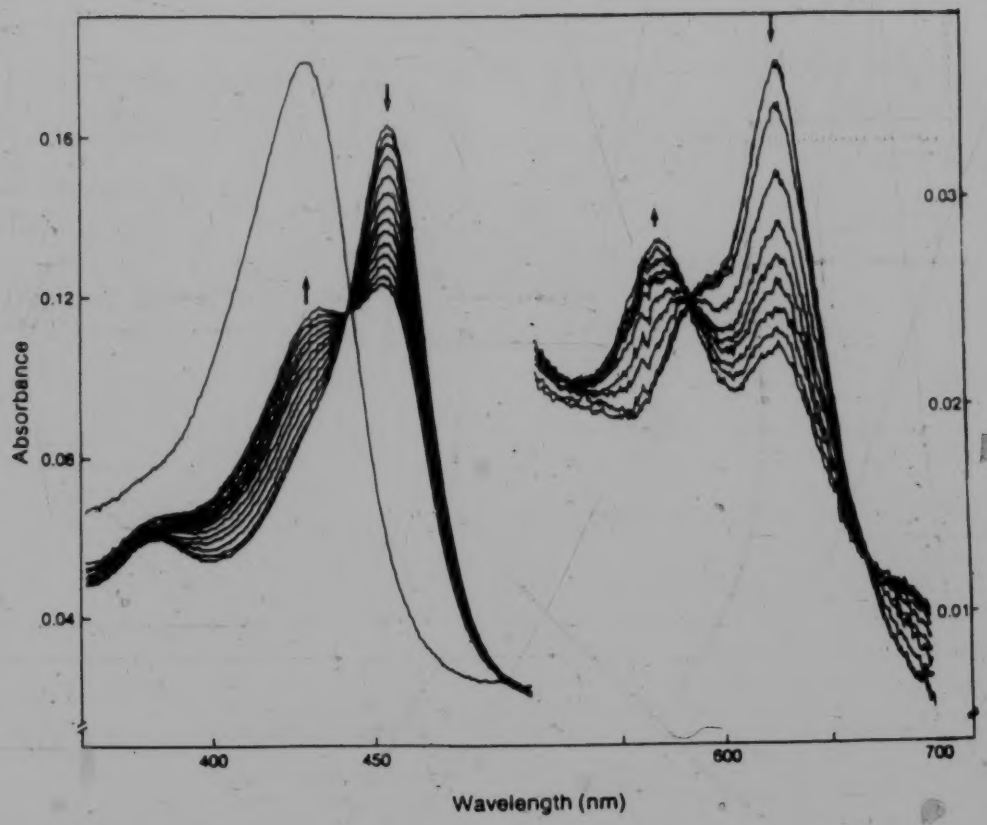

Figure 4.6. Spontaneous decay of compound II. Arrows show the direction of change of absorbance. Compound II was formed by adding 17 μM of H_2O_2 to 1.0 μM spleen myeloperoxidase in 0.1 M phosphate buffer, pH 7.1. Total scan time is approximately 62 minutes.

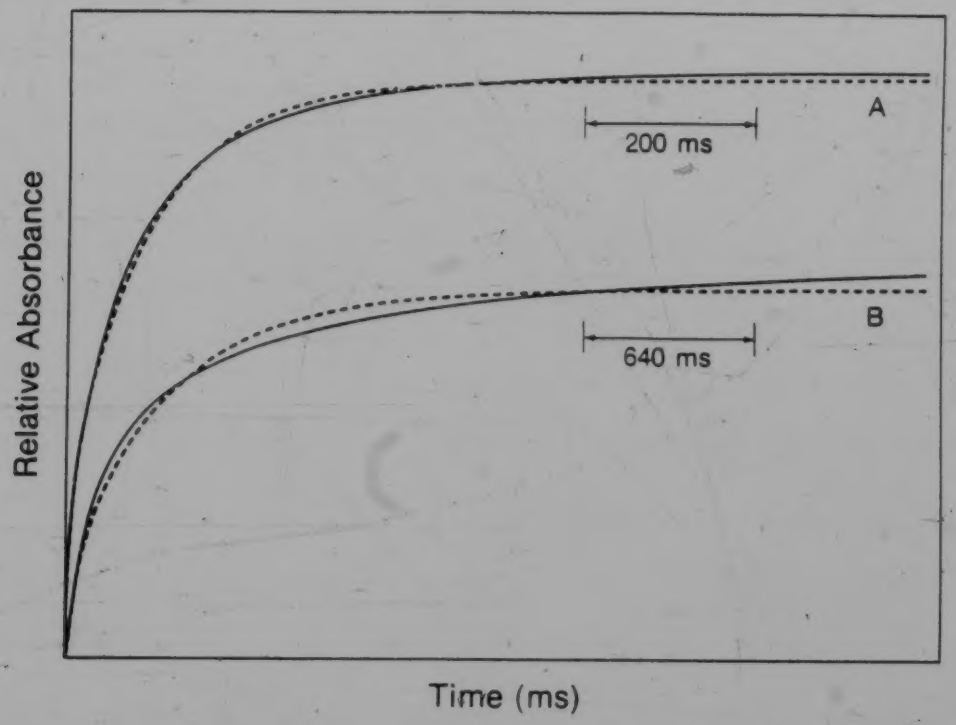

Figure 4.7. Typical experimental (——) and curve fit (-----) traces of compound II formation at (A) 120-fold excess H_2O_2 and (B) 10-fold excess of H_2O_2. Absorbance changes were followed at 455 nm. Final concentration of enzyme: 1.0 μM in 0.1 M phosphate buffer, pH 7.1.

enzyme the reaction is essentially monophasic. However, at a lower excess of hydrogen peroxide compound II formation proceeds with a slightly faster initial phase followed by a slower phase. When the initial portion of the curve was cut off, k_{obs} slightly decreased but fitted better in the plot of k_{obs} versus H_2O_2 concentration (Fig. 4.8). The apparent second order rate constant for compound II formation obtained from the plot of k_{obs} against $[H_2O_2]$ is $8.1 \pm 0.1 \times 10^4\ M^{-1}\ s^{-1}$.

The results shown in Fig. 4.7 suggest but do not prove conclusively that the reaction is biphasic. If indeed compound II formation is biphasic this could indicate a difference in the reactivity of the two hemes in myeloperoxidase; or it could be that the hemes exert a certain degree of negative cooperativity, i.e. when one heme is bound, the rate of binding to the other is reduced. These effects would be more pronounced at lower concentrations of hydrogen peroxide because at higher concentration the enzyme would be saturated with substrate and any difference in the rate of binding to the two hemes would be indistinguishable.

Table 4.1 shows a comparison between the ratio of k_{obs} for the initial phase and latter phase of the reaction when 10-fold and 5-fold

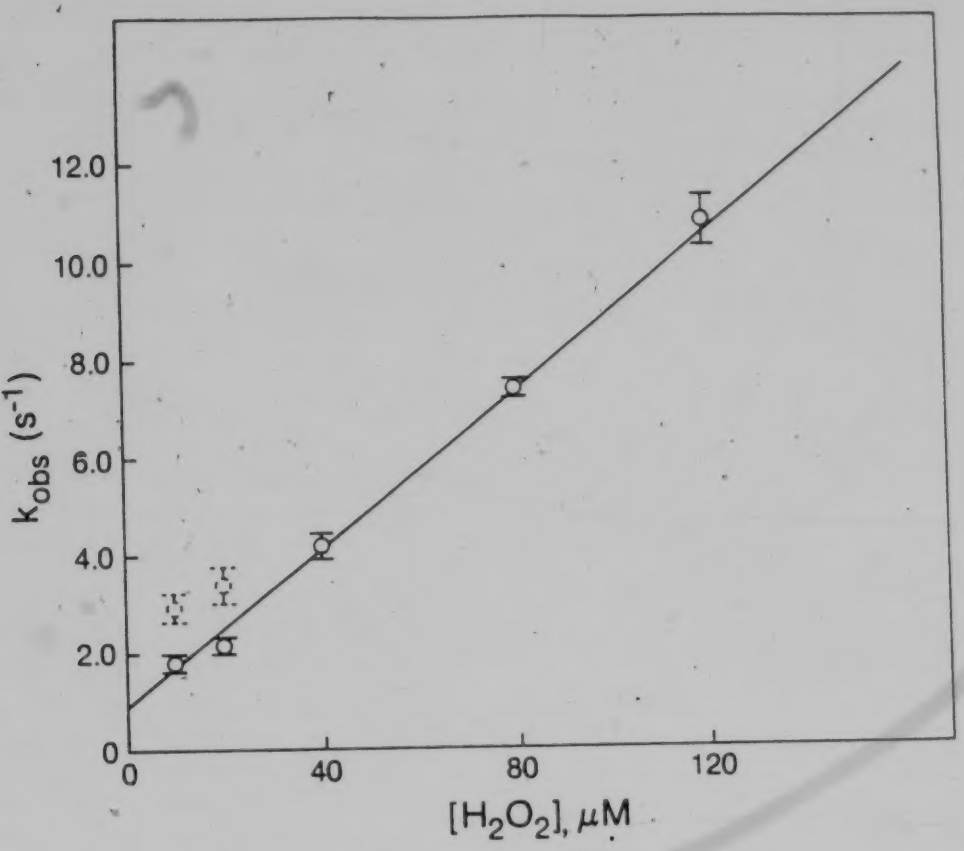

Figure 4.8. Pseudo-first order rate constants for compound II formation plotted against $[H_2O_2]$. The absorbance changes were followed at 455 nm. Final enzyme concentration: 1.0 μM in 0.1 M phosphate buffer, pH 7.1. The apparent second order rate constant obtained was $8.1 \pm 0.1 \times 10^4$ $M^{-1}s^{-1}$. For the first two points the initial portion of the reaction trace was cut off for the curve fit; the dashed circles represent k_{obs} values wherein the entire exponential trace of the reaction was considered.

Table 4.1. Ratio of k_{obs} for the initial and final phases of compound II formation at different $[H_2O_2]$. The final concentration of enzyme is 1.0 μM in 0.1 M phosphate buffer, pH 7.1.

excess $[H_2O_2]$	k_{obs} (s^{-1}) initial phase (0.5 s)	k_{obs} (s^{-1}) final phase (3 s)	initial/final
10X	6.16 ± 0.03	2.46 ± 0.06	2.5
5X	5.17 ± 0.34	1.64 ± 0.06	3.2

excess of H_2O_2 was used. If these differences are significant, then perhaps the hemes do exert negative cooperativity effects on each other or they have a slightly different reactivity with H_2O_2.

In conclusion, compounds I and II of spleen myeloperoxidase have been characterized using optical absorption spectroscopy. Despite myeloperoxidase's different prosthetic group, the spectra are similar to those observed for these intermediates in the more common peroxidases (horseradish peroxidase in particular). It takes a 20-fold excess of hydrogen peroxide for full formation of compound I which immediately decays to compound II. The rate constants for compounds I and II formation are reported. It is possible that the hemes exert negative cooperativity effects on each other or they have a slightly different reactivity to hydrogen peroxide.

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CHAPTER FIVE

REACTION OF MYELOPEROXIDASE COMPOUND II WITH ASCORBIC ACID

5.1. INTRODUCTION

Myeloperoxidase plays an important role in the host defense against invading microorganisms (1). It is present in polymorphonuclear leucocytes in exceptionally high concentrations; levels of 5% of the dry weight of the cell have been reported (2). *In vivo* , this enzyme converts Cl^- via a two-electron peroxidation step into hypochlorous acid, HOCl, which is a strong oxidizing agent capable of destroying microbes (3,4). Similarly, the myeloperoxidase-H_2O_2-I^- system mediates the oxidation of iodide by forming a reactive iodinating species which can iodinate and/or oxidize several compounds including proteins and lipids of bacterial membranes (5). Since the enzyme has such a significant physiological function it is important to determine what factors affect its microbicidal activity. It has been reported that ascorbic acid (Vitamin C) stimulates the chlorinating activity of human

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A version of this chapter has been submitted for publication.

Abbreviations: MPO, MPO-I, MPO-II, myeloperoxidase, compounds I and II, respectively; AH_2, ascorbic acid; $^{\cdot}AH$, ascorbate free radical.

myeloperoxidase (6). On the other hand, several reports state that iodination of bacteria engulfed by polymorphonuclear leukocytes was impaired by ascorbic acid (7-9). In order to explore the possible explanation of these contrary effects, it is necessary to understand the reaction mechanisms of myeloperoxidase with the appropriate substrates.

In the normal enzymatic cycle of peroxidases the native enzyme reacts with hydrogen peroxide to form the intermediate compound I in which the heme group contains two oxidizing equivalents more than the native enzyme. Compound I is reconverted into the native form via two one-electron steps in which two reducing substrate molecules are oxidized into radicals. During this reconversion another intermediate, compound II, is formed which contains one oxidizing equivalent more than the native enzyme (10). Taking myeloperoxidase (MPO) as an example, the enzymatic cycle can be represented by Equations 5.1-5.3:

[5.1] $MPO + H_2O_2 \longrightarrow MPO\text{-}I + H_2O$

[5.2] $MPO\text{-}I + AH \longrightarrow MPO\text{-}II + \cdot AH$

[5.3] $MPO\text{-}II + AH \longrightarrow MPO + \cdot AH$

The peroxidation of chloride catalyzed by myeloperoxidase differs

from the above mechanism in that compound I is reconverted into native enzyme in a one two-electron reduction step (Equation 5.4) (11).

[5.4] $$MPO\text{-}I + Cl^- \longrightarrow MPO + HOCl$$

During turnover, compound II is also formed,apparently by extraneous reactions, but it is inactive in the chlorination reaction (12). It has been proposed that ascorbic acid stimulates the chlorinating activity of myeloperoxidase by converting compound II into native enzyme which again participates in the formation of hypochlorous acid (6). However, no mechanistic explanation has been offered for the inhibitory effect of ascorbic acid on the iodination reaction (7-9).

In this chapter rapid scan spectral traces the course of the reaction of compound II of myeloperoxidase with ascorbic acid are shown. The reaction follows saturation kinetics and the pH-rate profile suggests that the optimum pH for the reaction lies at around pH 4.5. At this optimum pH the activation energy for the reaction is 13.2 kJ mol^{-1}. A comparison of the spectral scans, kinetic parameters and pH-rate profiles for the reaction indicates that myeloperoxidases from different sources namely, canine pus, bovine spleen and human neutrophil behave

similarly. I^- reacts with compound II of myeloperoxidase with a rate comparable with that of ascorbic acid; a plausible explanation of the inhibitory effect of ascorbic acid on the iodination reaction is given.

5.2. MATERIALS AND METHODS

Bovine spleen myeloperoxidase was isolated and purified using the procedure described in Chapter 2. Canine myeloperoxidase was obtained as previously described (13). Human neutrophil myeloperoxidase was obtained by suspending buffy coats of outdated human blood in buffer containing 10 mM Tris, 0.5 M NaCl, 5 mM $CaCl_2$ at pH 7.5. The buffer also contained 50 μM $ZnSO_4$ and 0.05% Brij-35. The buffy coat suspension was lysed using a Virtis homogenizer. Three freeze-thawing cycles were used to release myeloperoxidase from the granules. The resulting extract was centrifuged to remove insoluble material and the supernatant was applied to a Reactive Red-120 agarose (Sigma Chemical Co.) column and washed with the same buffer to remove the majority of the hemoglobin. The NaCl concentration of the elution buffer was increased to 1 M to desorb other impurities, after which the myeloperoxidase was eluted by increasing the NaCl concentration to 4 M.

This fraction was concentrated 50-fold with a PM-10 membrane (Amicon) then the ionic strength was adjusted to 0.1 M. The enzyme was further purified by ion exchange chromatography through CM-Sepharose CL-6B (Pharmacia) followed by gel filtration through Sephacryl S-200 (Pharmacia). The enzyme preparations used in this study exhibited Rz values (A_{430}/A_{280}) of 0.80 or greater. The myeloperoxidase concentration was determined spectrophotometrically using an extinction coefficient at 430 nm of 178 mM^{-1} cm^{-1} per molecule of enzyme (14).

L-ascorbic acid (Sigma) and potassium iodide (BDH Chemicals) were reagent grade and used without further purification. Aqueous solutions were freshly prepared before each day experiments were performed. Hydrogen peroxide (≈30% solution, BDH Chemicals) concentration was determined after appropriate dilution using the horseradish peroxidase assay (15). Chemicals used for the buffers were reagent grade and used without further purification. Solutions were prepared using deionized water obtained from the Milli Q system (Millipore).

All experiments were carried out at 25 ± 0.5 °C except as otherwise indicated and an ionic strength of 0.1 M due entirely to the

contribution of the buffer. The following buffers were used: citrate-phosphate (pH 4.0-5.5) and phosphate (pH 6.0- 8.0). The pH was measured using a Fisher microprobe electrode and a Fisher digital pH meter.

Optical absorption spectra were obtained with a Cary 219 recording spectrophotometer. Rapid scan spectra were recorded using the Photal (formerly Union Giken) RA 601 Rapid Reaction Analyzer equipped with a 1-cm observation cell. The absorption spectra were measured by means of a multichannel photodiode array and memorized in a digital computer system (Sord M200 Mark II). The analog replica was plotted on an X-Y recorder. Spectra were scanned from 400-500 nm. Kinetic measurements were conducted using the Photal RA 601 in the stopped flow mode. One reservoir contained 1 μM enzyme in 0.2 M (ionic strength) buffer and varying concentrations of ascorbic acid (100-600 μM); the other reservoir contained 20 μM H_2O_2 in aqueous solution. The reaction was followed at 455 nm, the isosbestic point of native enzyme and compound I and where the maximum change in absorbance is observed upon conversion of compound II to native enzyme. Pseudo-first order rate constants were determined by a nonlinear least squares computer analysis of exponential traces.

5.3. RESULTS

Figure 5.1A shows the formation of compound II from native enzyme in the presence of hydrogen peroxide and ascorbic acid. The Soret peak at 430 nm decreases and one appears at 455 nm. Full formation of compound II was achieved in about 2 s using 20 equivalents of H_2O_2.

Spectra taken at shorter times after mixing (data not shown) actually show the initial formation of compound I which very rapidly goes to compound II. Native enzyme and compound II exhibit an isosbestic point at about 440 nm. In the absence of ascorbic acid, compound II is stable for about 10 minutes. However, in the presence of ascorbic acid, it converts back to native form within 1.5 s (Fig. 5.1B) with an isosbestic point at 440 nm.

Figure 5.2 shows a typical trace of the reaction of myeloperoxidase and H_2O_2 in the presence of ascorbic acid followed at 455 nm. The biphasic curve shows the initial formation of compound II and then its subsequent reaction with ascorbic acid causing an exponential decrease in the absorbance. From the computer exponential curve-fit, pseudo-first order rate constants, k_{obs}, for the compound II reaction with ascorbic acid were obtained from the latter part of the

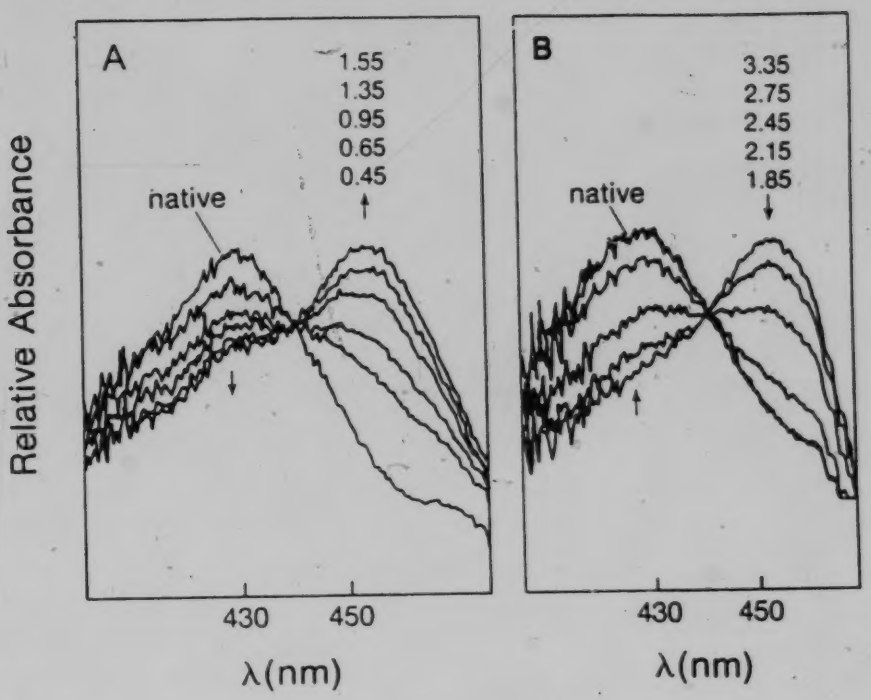


Figure 5.1. Formation of MPO II and return to native enzyme. One reservoir contained 1 μM bovine spleen MPO in 0.2 M phosphate buffer, pH 7.1 and 300 μM ascorbic acid. The other reservoir contained 20 μM H_2O_2 in aqueous solution. A) Spectral scans showing the formation of MPO II. B) Conversion of MPO II to native enzyme. Arrows indicate direction of absorbance changes. Times are in s after mixing.

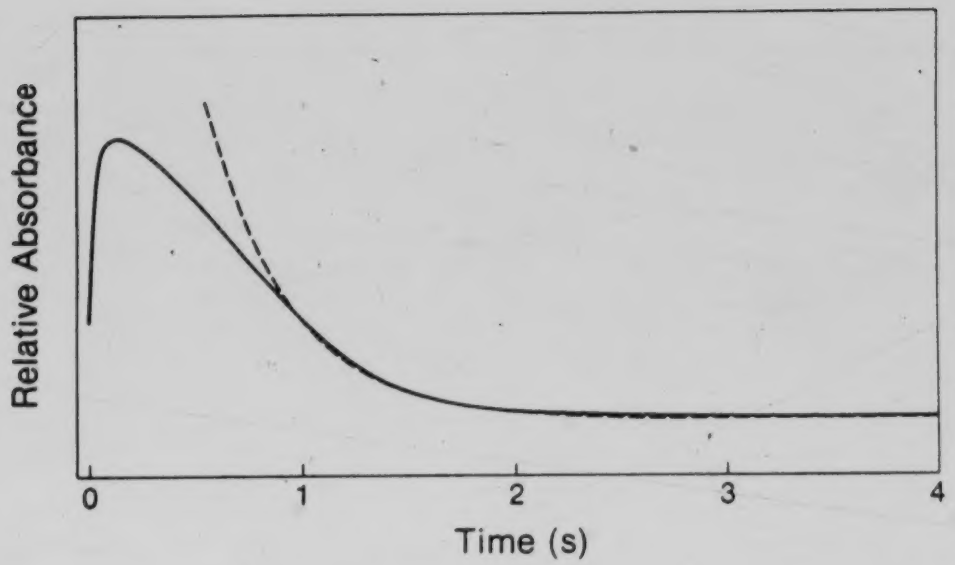


Figure 5.2. Typical time course of the reaction between bovine spleen myeloperoxidase, H_2O_2 and ascorbic acid. Absorbance changes were followed at 455 nm, the isosbestic point between native MPO and MPO I. The initial increase in absorbance is due to the formation of MPO II. MPO II then reacts with ascorbic acid and goes back to native form. The broken line is an exponential curve fit.

curve.

The plot of k_{obs} versus concentration of ascorbic acid is a rectangular hyperbola. The plot levels off at high ascorbic acid concentrations (Fig. 5.3). This behavior can be explained by a simple binding interaction between reactants according to the following equation:

$$\text{[5.5]} \qquad \text{MPO-II} + AH_2 \underset{}{\overset{K_1}{\rightleftharpoons}} \text{MPO-II-}AH_2 \xrightarrow{k_1} \text{MPO} + \cdot AH$$

which leads to:

$$\text{[5.6]} \qquad k_{obs} = \frac{k_1}{1 + K_1/[AH_2]}$$

where k_1 is a first order rate constant (s^{-1}) and K_1 (M) is an apparent dissociation constant given by:

$$\text{[5.7]} \qquad K_1 = \frac{[\text{MPO-II}]\,[AH_2]}{[\text{MPO-II-}AH_2]}$$

The constants k_1 and K_1 were calculated directly from Equation 5.6 using a non-linear least squares fit to the data.

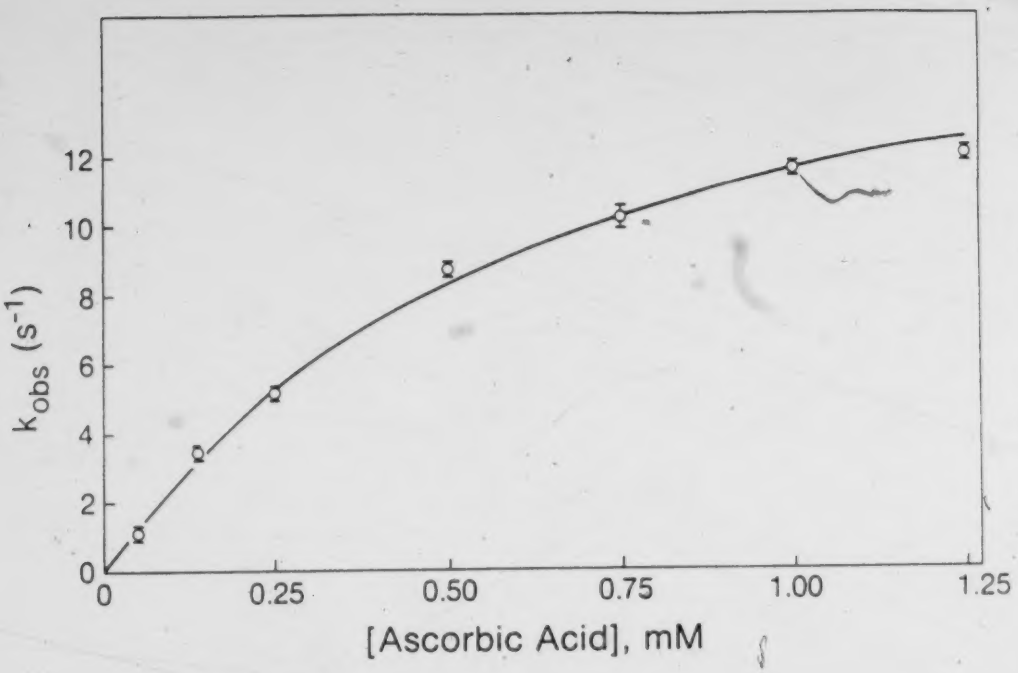


Figure 5.3. Reaction of MPO II with ascorbic acid. One reservoir contained 1 μM bovine spleen MPO in 0.2 M phosphate buffer, pH 7.1 and different concentrations of ascorbic acid. The other reservoir contained 20 μM H_2O_2 in aqueous solution. k_{obs} was determined from the exponential decrease in absorbance at 455 nm. The plot is a nonlinear least squares fit of the data. Equation 5.6 in the text was used for the curve fit.

Similar plots of k_{obs} versus ascorbate concentration were obtained whether canine myeloperoxidase, human neutrophil myeloperoxidase or bovine spleen myeloperoxidase was used. Table 5.1 shows a comparison of the kinetic parameters, k_1 and K_1, for the reaction of compound II of myeloperoxidase from the three different sources with ascorbic acid.

The dependence of k_{obs} upon pH is shown in Fig. 5.4. The plot of k_{app} versus pH shows a similar trend. The myeloperoxidase used to obtain Fig. 5.4 is from the human source but all three myeloperoxidases exhibited a similar pH-rate profile. The k_{obs} decreased at extremes of pH and reached its maximum value at about pH 4.5.

At lower ascorbic acid concentrations, i.e. < 250 μM, the plot of k_{obs} versus ascorbic acid concentration is linear from which an apparent second order rate constant (k_{app}) is obtained from the slope. The temperature dependence of k_{app} was measured over the temperature range 9.0-30.5 ^{o}C. Figure 5.5 is an Arrhenius plot from which the activation energy was calculated to be 13.2 ± 1.0 kJ mol^{-1}.

Table 5.1. Kinetic parameters for the reaction of compound II of myeloperoxidase from different sources with ascorbic acid. The values were obtained by measuring the k_{obs} for various ascorbic acid concentration. Data was analyzed using nonlinear least squares calculations.

Source	k_1 (s^{-1})	K_1 (M)
canine pus	(A) 17.5 ± 1.7	(A) 2.0 ± 0.3 x 10^{-3}
bovine spleen	(A) 18.8 ± 1.3	(A) 6.3 ± 0.9 x 10^{-4}
human neutrophil	(A) 12.6 ± 0.6	(A) 6.6 ± 0.4 x 10^{-4}

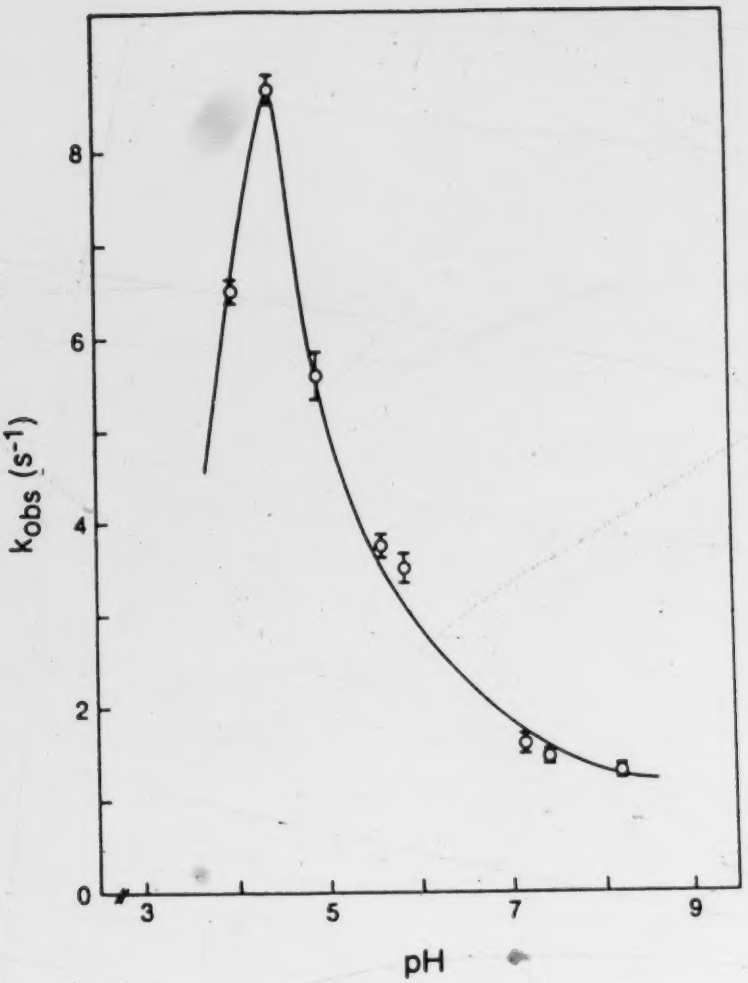


Figure 5.4. pH dependence of human neutrophil MPO II reaction with ascorbic acid. Experiments and k_{obs} determinations were conducted as in Fig. 5.3. Final concentrations: 0.5 μM MPO, 10 μM H_2O_2, 0.1 mM ascorbic acid. Buffers used were citrate-phosphate (pH 4.0-5.5) and phosphate (pH 6-8). The rate was measured at 25.5 ^{o}C and ionic strength of 0.1 M.

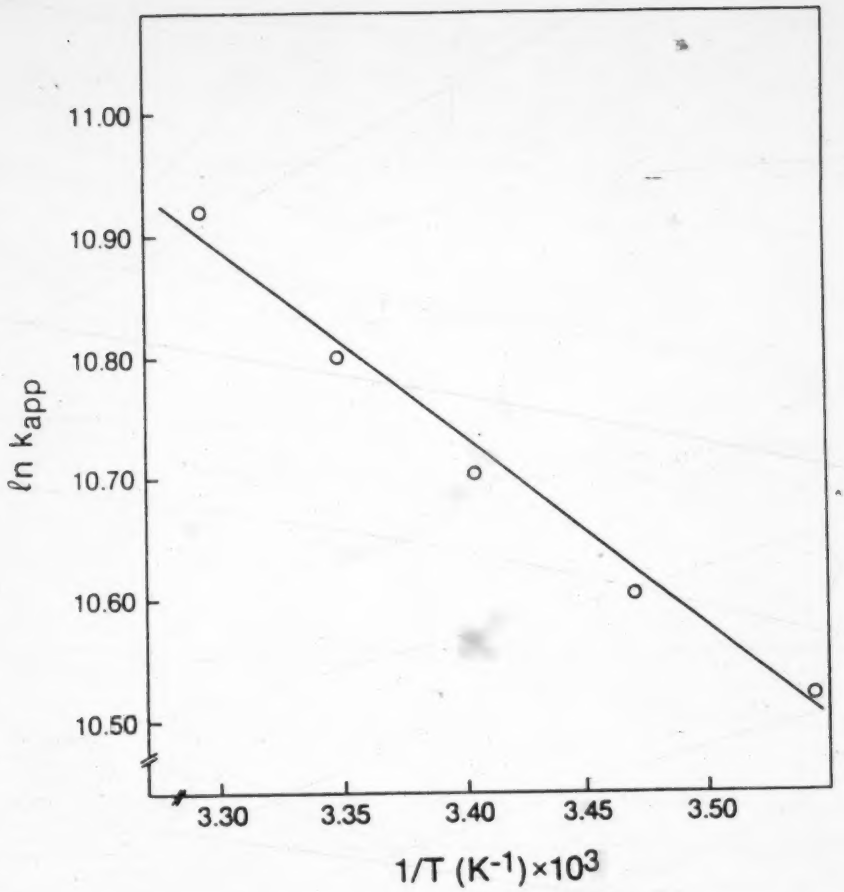


Figure 5.5. Arrhenius plot for canine MPO II reaction with ascorbic acid. Values of k_{app} were obtained as described in the text. The temperature was varied from 9.0 to 30.5 ^{o}C. The buffer used was citrate-phosphate, pH 4.8, ionic strength 0.1 M.

Figure 5.6 shows the reaction of compound II of bovine spleen enzyme with iodide. Similar results were obtained for the myeloperoxidases from canine and human sources. (A) is the spectrum of the native enzyme and (B) is the spectrum of compound II formed by adding a 20-equivalent excess of H_2O_2 to native enzyme. Addition of excess iodide to native enzyme caused a decrease in absorption coefficient at 430 nm (C). When 20 equivalents H_2O_2 were added to this enzyme-iodide complex the formation of compound II could not be detected during the scan period because it rapidly converted back to native enzyme. The final spectrum (D) overlaps with (B) due to the binding of the native enzyme with excess iodide in the medium.

Pseudo-first order rate constants for the reaction between bovine spleen compound II and iodide were obtained as in the ascorbic acid reaction. The plot of k_{obs} versus concentration of iodide (Fig. 5.7) similarly shows that the reaction follows saturation kinetics. The parameters k_1 and K_1 were found to be $(13.6 \pm 0.8)\ s^{-1}$ and $(1.1 \pm 0.1 \times 10^{-4})$ M, respectively.

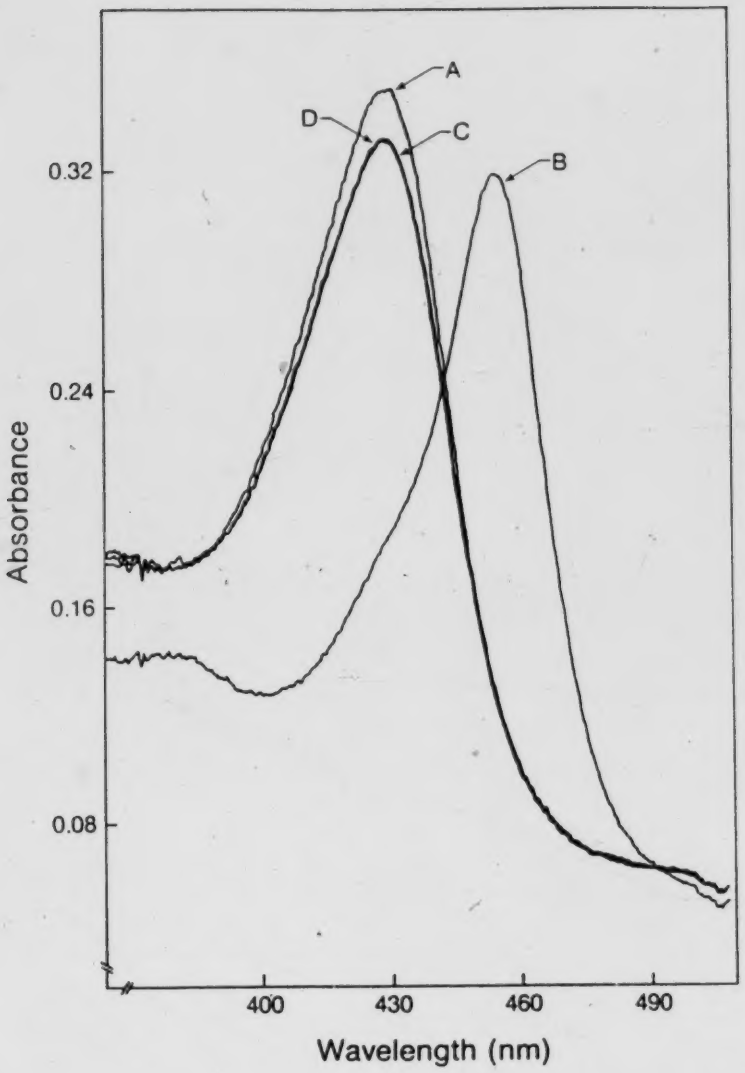


Figure 5.6. Reaction of spleen MPO II with iodide. A) The reaction cuvette contained 2 μM spleen MPO in 0.1M phosphate buffer, pH 7.1. B) 40 μM H_2O_2 was added to A. C) Spectra obtained when 100 μM KI was added to A. D)40 μM H_2O_2 was added to C. Total scan time: 90 s.

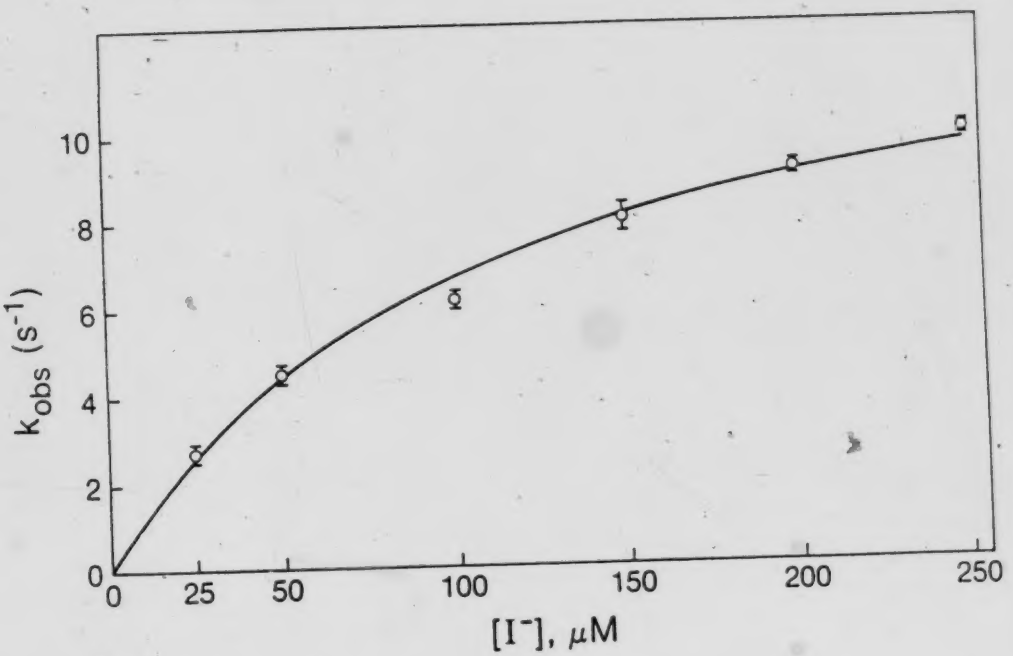


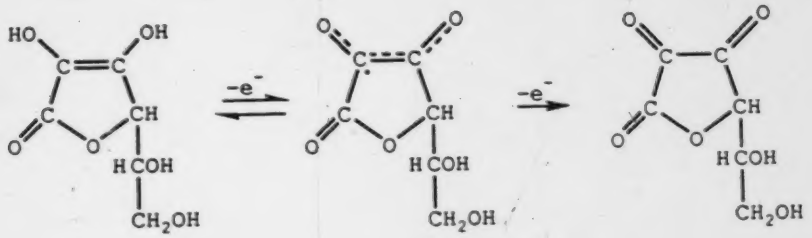
Figure 5. 7. Plot of pseudo-first order rate constants versus concentration for the reaction between MPO II and iodide. Experimental conditions and calculations are the same as in Fig. 5.3 except that iodide was used instead of ascorbic acid.

5.4. DISCUSSION

Ascorbic acid has been implicated in various physiological functions. It is widely distributed throughout the tissues but high levels are found in the liver, spleen, brain, blood, and adrenal and pituitary glands (16,17). It is important in increasing iron absorption, in collagen synthesis and as a biological blocking agent against nitrosamine formation (18). Ascorbic acid also functions as an antioxidant by protecting double bonds by scavenging oxygen and other radicals (19,20).

Ascorbic acid is a dibasic acid (pK_{a1}= 4.2; pK_{a2} =11.3) with a bifunctional ene-diol group built into a heterocyclic lactone ring. Its principal pathway of oxidation is given by Eqn. 5.8 (20):

[5.8]



ascorbic acid | ascorbate free radical | dehydroascorbic acid

Polymorphonuclear leukocytes contain a high level of ascorbate,

approximately 0.7 mM (21). Presently available evidence suggests that ascorbic acid is a stimulant of leucocyte functions. Human leucocytes take up ascorbic acid as dehydroascorbic acid and reduce it promptly to ascorbic acid inside the cell (22). During phagocytosis, the ascorbate content was found to decrease because of its oxidation to dehydroascorbate (23). This decrease in ascorbate concentration can be attributed to ascorbic acid's participation in the myeloperoxidase enzymatic cycle. As shown in Eqn. 5.4 the antimicrobial activity of myeloperoxidase has been mainly attributed to the ability of compound I to oxidize Cl^- to HOCl, a powerful oxidizing agent. In the enzymatic cycle another intermediate, compound II, which is inactive in the chlorination reaction, is also formed. Compound II accumulates during turnover because its slow spontaneous decay to native enzyme takes about an hour (data not shown). However, in the presence of 300 μM excess of ascorbic acid the quantitative conversion to native enzyme takes place in 1.5 s. The reaction between compound II and ascorbic acid is clearly shown by the spectral scans (Figs. 5.1A and B). An isosbestic point at 440 nm indicates that compound II does not pass through any other intermediate when it goes back to native enzyme.

It was necessary to use an excess of H_2O_2 to ensure the complete formation of compound II. The plot of k_{obs} versus ascorbic acid concentration was nonlinear (Fig. 5.3). Nonlinear saturation responses have been observed previously in the reactions of compounds I and II of manganese peroxidase with Mn^{II} (24). This was attributed to a simple binding interaction between the enzyme and substrate followed by the enzyme reduction; the same explanation is offered for the present results. The kinetic parameters obtained using myeloperoxidase from canine pus, human neutrophil and bovine spleen are comparable (Table 5.1). This lends further substance to the claim that the previously called "spleen green heme protein" is in fact myeloperoxidase (25-29).

The pH rate profile shows that the compound II-ascorbic acid reaction reaches an optimum at pH=4.5. The drop in the rate at lower pH may be caused by the presence of a group in the enzyme which when protonated prevents the reaction of the enzyme with H_2O_2, thus leading to a lower initial concentration of compound II (30,31). Attempts to study the reaction at lower pH gave nonreproducible results presumably due to partial denaturation of the enzyme. At pH values above 7, the rate

leveled off. Apparently,compound II is less reactive with ascorbic acid at high pH. This observation is consistent with the reported stability of compound II due to a heme-linked ionization of a distal amino acid residue (32).

It is interesting to note that although the myeloperoxidase-H_2O_2-Cl^- system can function over a wide pH range, studies reveal that an optimum is obtained at about pH 5 (33,34) which is very close to the optimum observed for the compound II-ascorbic acid reaction. Moreover, it has been observed that a decrease in the pH to 4 occurs in the phagocytic vacuole following phagocytosis (35). Thus, the stimulating effect of ascorbic acid on the chlorination reaction reasonably reaches its peak at pH 4.5, a value similar to what was found in this work.

An intriguing observation made several years ago (7,9) is that high concentrations of ascorbate in polymorphonuclear leucocytes impair bacterial iodination. Why would ascorbic acid stimulate the myeloperoxidase-catalyzed chlorination reaction and impair the iodination reaction?

While both iodide and chloride are oxidized by myeloperoxidase compound I, only iodide can be oxidized by compound II. Spectral and

kinetic studies show that the rate of reduction of compound II to native is the same in the presence and in the absence of chloride. On the other hand, iodide hastens the conversion of compound II to native state. In the presence of chloride and ascorbic acid, the compound II which forms during turnover is converted back to native enzyme which again forms the chlorinating species HOCl via compound I, thereby resulting in an increase in the chlorinating activity. On the other hand, in the presence of iodide and ascorbic acid, the iodinating species formed when compound II reacts with iodide is decreased because ascorbic acid competes for the binding site. Both iodide and ascorbic acid form a complex with compound II with comparable dissociation constants; moreover, the rate of breakdown of the complex to form the products are of the same order of magnitude. This competition probably caused the observed decrease in iodination of bacteria engulfed by polymorphonuclear leucocytes.

Although myeloperoxidase-H_2O_2-I^-constitute a bactericidal system, bacterial killing *in vivo* probably involves chloride rather than iodide. This is chiefly because chloride is present in the cell in concentrations considerably greater than iodide (36,37). This is further

supported by the observation that ascorbate added to suspending media impairs the iodination of bacteria engulfed by polymorphonuclear leucocytes but has no effect on bacterial killing (8).

How much ascorbate is required for stimulating the chlorinating activity of myeloperoxidase? The ascorbate level in polymorphonuclear leucocyte is approximately 0.7 mM (21) at which concentration the compound II- ascorbic acid reaction rate approaches saturation. Thus, it makes little, or no, pharmacological sense to load the human system with excess ascorbic acid. However, massive doses of Vitamin C (up to 10 g/day) also do not constitute a health risk since man has a limited intestinal absorption capacity for ascorbic acid and a very efficient elimination via the kidney (38).

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CHAPTER SIX

REACTION OF MYELOPEROXIDASE COMPOUND III WITH ASCORBIC ACID

6.1. INTRODUCTION

Myeloperoxidase, like other peroxidases, is known to exist in several spectroscopically distinguishable forms: ferric native enzyme and the oxidized intermediates: compounds I, II and III (1-3). In the presence of H_2O_2, myeloperoxidase catalyzes the oxidation of Cl^- to HOCl, which acts as a bactericidal agent. HOCl formation is thought to proceed via a peroxidase mechanism in which H_2O_2 reacts with native enzyme to give compound I, which in turn reacts with Cl^- to give HOCl (4,5). During turnover, the reaction of compound I with other electron donors may result in the formation of compound II. In addition, native enzyme can undergo reaction with $O_2^{-\cdot}$, which is also present in phagocytes (6,7), to give compound III. Both compounds II and III are inactive in chloride peroxidation (8-10). Thus, the accumulation of these two intermediates limits the microbicidal activity of the enzyme.

A version of this chapter has been submitted for publication.

Abbreviations: DHF, dihydroxyfumarate; HRP, horseradish peroxidase; MPO, myeloperoxidase; SOD, superoxide dismutase; AH^-, ascorbate.

In the previous chapter, it was discussed that ascorbic acid enhances the chlorinating activity of myeloperoxidase by increasing the turnover of compound II to native enzyme. Here ascorbic acid is shown to also reduce compound III to the ferric form, thus making it available for HOCl formation. Furthermore, the preparation of a more stable and relatively pure compound III of bovine spleen myeloperoxidase using the oxidation of dihydroxyfumarate (DHF) is reported. This method was originally used with HRP (11). The effect of pH on the formation and decay of compound III is presented. The possibility that compound III could function as a superoxide dismutase is also considered.

6.2. MATERIALS AND METHODS

Bovine spleen myeloperoxidase was isolated and purified using the procedure described in Chapter 2. The enzyme preparations used in this study exhibited Rz values (A_{430}/A_{280}) of 0.80 or greater. The myeloperoxidase concentration was determined spectrophotometrically using an extinction coefficient at 430 nm of 178 $mM^{-1}cm^{-1}$ per molecule of enzyme (12). Superoxide dismutase (Sigma) was used without further purification. Its concentration was calculated using an absorbance

coefficient of 15.9 mM^{-1} cm^{-1} at 265 nm (13). Xanthine and xanthine oxidase (0.025 units/mL) were also purchased from Sigma.

L-ascorbic acid and DHF (Sigma) were reagent grade. Aqueous solutions were freshly prepared before each day experiments were performed. DHF was dissolved in Ar-saturated water immediately prior to use. Hydrogen peroxide (≈30% solution, BDH Chemicals) concentration was determined using the horseradish peroxidase assay (14). All chemicals used for the buffers were reagent grade and used without further purification. Solutions were prepared using deionized water obtained from the Milli Q system (Millipore).

All experiments were carried out at 25.0 ± 0.5°C and an ionic strength of 0.1 M due entirely to the contribution of the buffer. The pH was measured using a Fisher microprobe electrode and a Fisher digital pH meter.

Optical absorption spectra were obtained with a Cary 219 recording spectrophotometer. Rapid scan spectral and kinetic measurements were conducted using the Photal (formerly Union Giken) RA 601 Rapid Reaction Analyzer equipped with a 1-cm observation cell. One reservoir contained a stable preparation of compound III (approximately 1 μM) in 0.2 M (ionic

strength) phosphate buffer and the other reservoir contained ascorbic acid in aqueous solution. Pseudo-first order rate constants were determined by a non-linear least squares computer analysis of exponential traces.

6.3. RESULTS

Compound III of myeloperoxidase from bovine spleen was prepared by reacting the ferric enzyme with $O_2^{-\cdot}$ generated from both a) aerobic oxidation of DHF and b) the xanthine-xanthine oxidase reaction system. Fig. 6.1 shows the visible spectra of compound III formed using the DHF reaction system. The Soret peak of the native enzyme disappears and a new one at 450 nm appears. Also, the broad shoulder at 625 nm becomes a sharp peak.

Fig. 6.2 shows that compound III formation was not instantaneous. Moreover, a large excess (100-fold) of DHF was required for full formation of this enzyme intermediate. The order of addition of DHF and H_2O_2 to the native enzyme had no effect on the amount nor the rate of compound III formation.

The compound III prepared using DHF was very stable as shown by

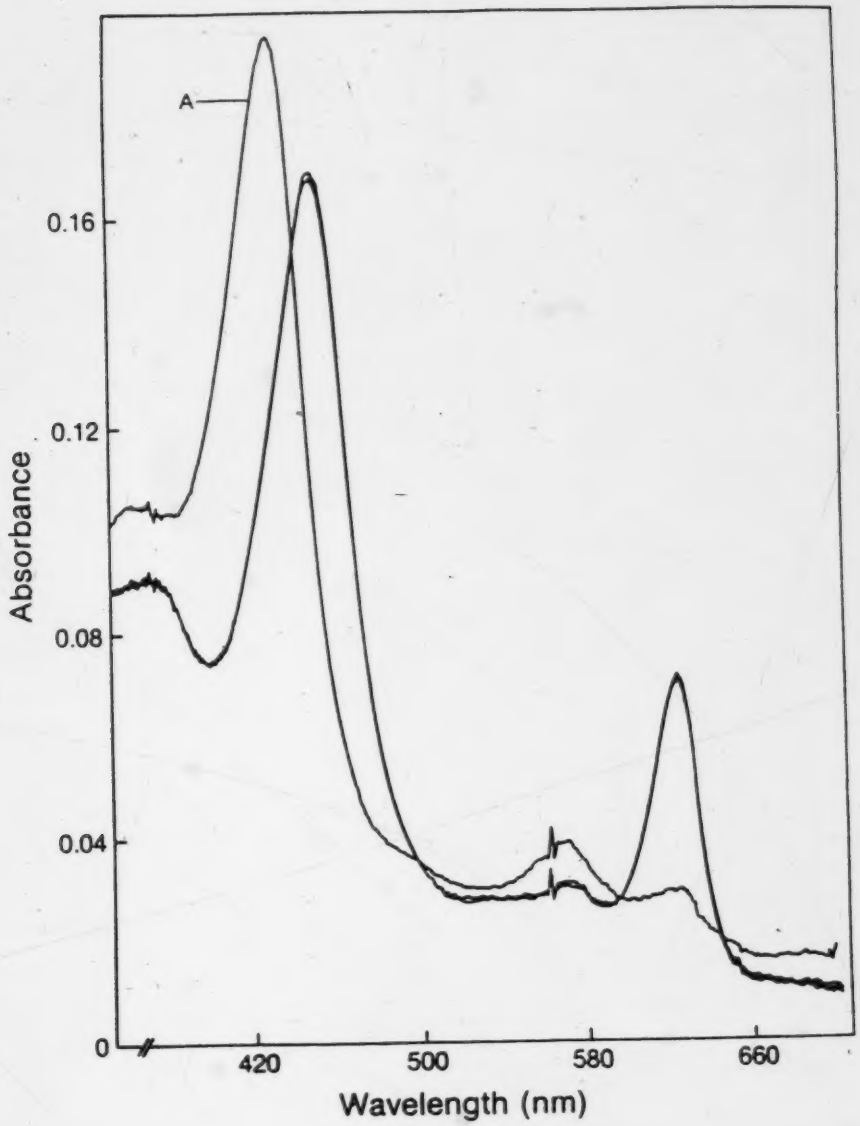

Figure 6.1. Spectra of compound III formed using the DHF system. A) Native enzyme: The reaction cuvette contains 1.1 μM of spleen myeloperoxidase in 0.1 M phosphate buffer, pH 7.1.B) Compound III: 100 μM DHF and 1 μM H_2O_2 was added to the native enzyme in A.

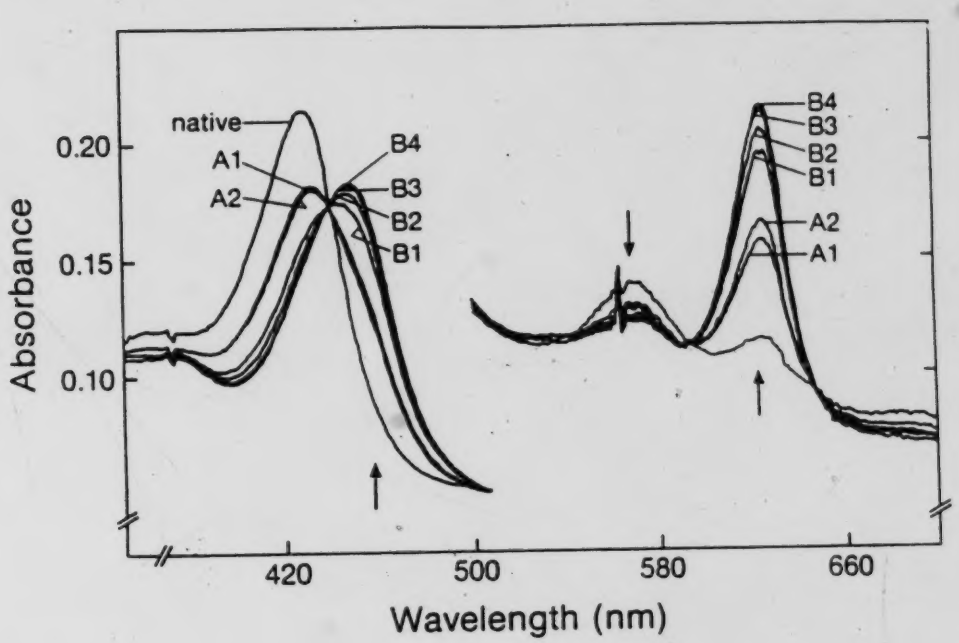

Figure 6.2. Effect of amount of DHF added on compound III formation.

A) To 1.2 μM native enzyme in 0.1 M phosphate buffer, pH 7.1, was added 62 μM of DHF and 1 μM H_2O_2. Spectra 1 and 2 were taken 3 min 30 s after each other. B) 38 μM more DHF was added to A. Scan times for spectra 1,2,3 and 4 are the same as in A. The arrows indicate the direction of absorbance changes with increasing time.

the decay spectra in Fig. 6.3. For the first 100 s (scans 1-8), the absorbances at 450 and 625 nm steadily decrease but there was virtually no increase in the absorbances at 430 and 570 nm to suggest formation of the native enzyme. Later on however, the decay of compound III was accompanied by formation of the native enzyme, the scans now exhibiting an isosbestic point at 440 nm. When 5 μM more H_2O_2 was added to compound III there was an initial increase in the absorbances at 450 and 625 nm. A larger excess (50 μM) of H_2O_2 caused a decrease in absorbance at these wavelengths with no concommitant increase at 430 and 570 nm.

The stability of compound III prepared using the DHF system was compared with that using the xanthine/xanthine-oxidase system. Compound III prepared using the latter method exhibited a broad band at about 446 nm, remained stable only for about 10 minutes, then started to decay back to the native state with a well-defined isosbestic point at 440 nm (Fig. 6.4).

Fig. 6.5 shows the effect of pH on the stability of compound III. At pH 4.7 compound III remains virtually unchanged during 15 min while at pH 9.2 considerable reversion back to the native state has occurred within the same time span.

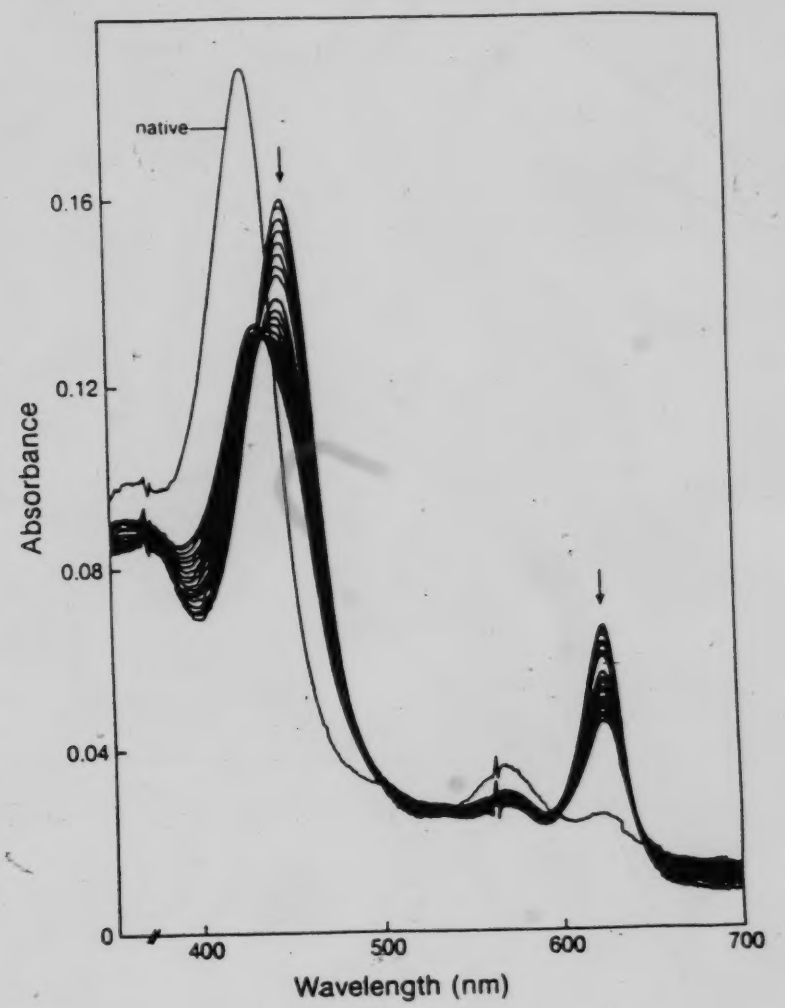

Figure 6.3. Spectral changes accompanying the decay of compound III formed using DHF. Compound III was prepared as described in Fig. 6.1. Each scan takes 12 min 30 s. The last scan was taken after 4 hrs and 10 min. The arrows indicate the direction of absorbance changes with increasing time.

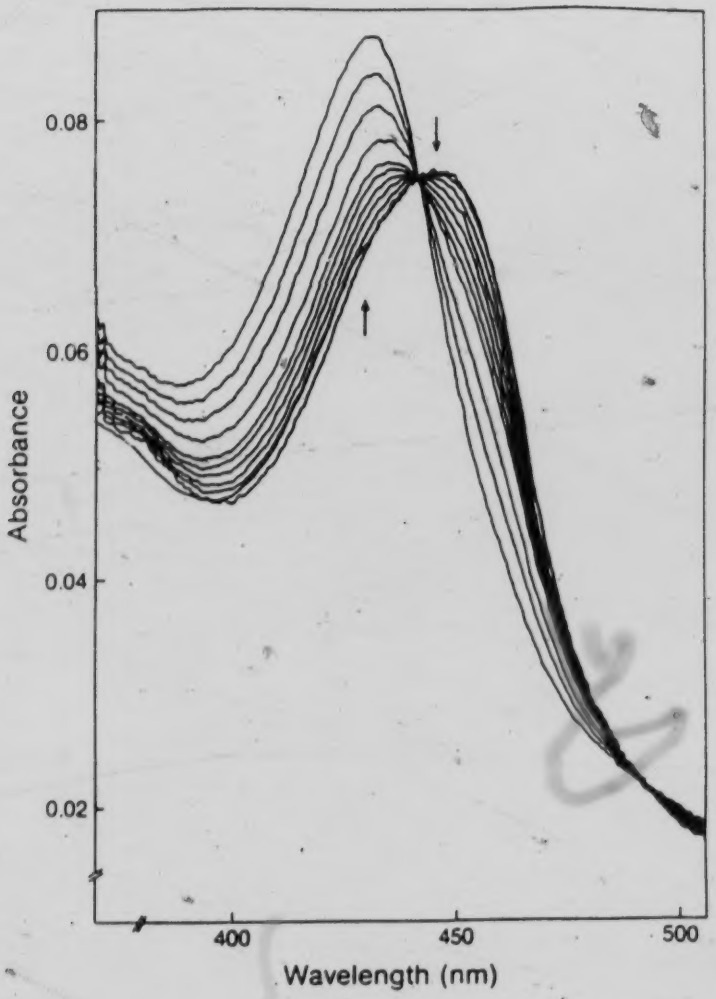

Figure 6.4. Spectral changes accompanying the decay of compound III formed using the xanthine/xanthine oxidase system. Compound III was formed by adding 10 µL of xanthine oxidase to a reaction mixture containing 1 µM myeloperoxidase, 0.5 mM xanthine, 0.1 M phosphate buffer, pH 7.1. The first scan was started 10 minutes and the last scan 75 minutes after initiation of the reaction. The arrows indicate the direction of absorbance changes with increasing time.

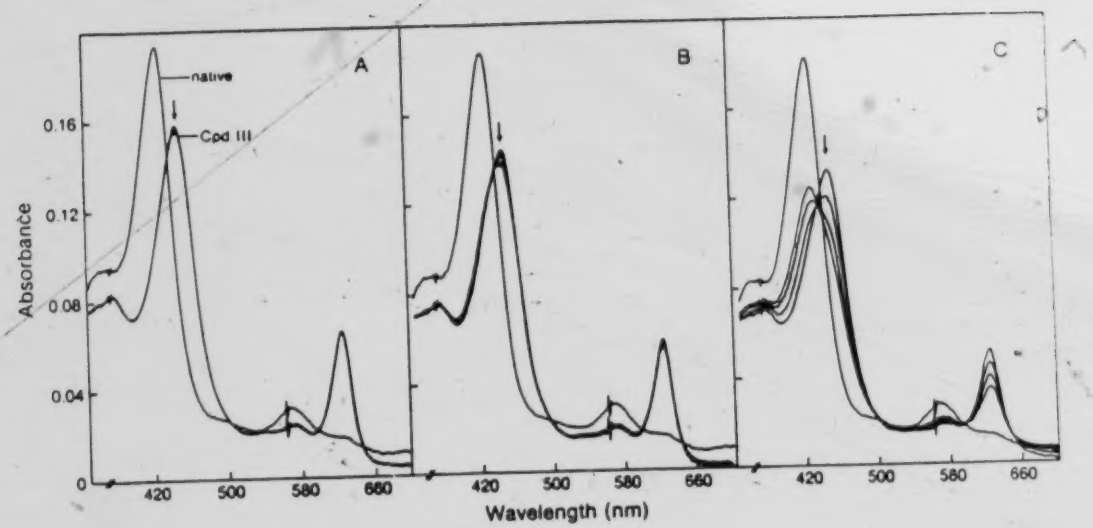

Figure 6.5. Effect of pH on the stability of compound III. Compound III was prepared by adding 100 μM DHF and 1 μM H_2O_2 to 1 μM spleen myeloperoxidase in 0.1 M buffer. Repetitive scans were taken for a period of 3 min 30 s each. A) citrate-phosphate buffer, pH 4.7. B) phosphate buffer, pH 7.1. C) bicarbonate buffer, pH 9.2.

The addition of SOD to compound III caused an acceleration of its decay to the ferric form (Fig. 6.6). The isosbestic point at 440 nm was maintained. When 6.5 μM SOD was added to 1μM native enzyme prior to the addition of DHF and H_2O_2, compound III formation was completely inhibited. However, when a lower concentration of SOD was added (i.e. 3 μM), some compound III still formed.

In an attempt to accelerate the decay of compound III to the ferric state, ascorbic acid, which is both a reducing agent and a free radical scavenger, was added to the reaction system. Fig. 6.7 shows rapid scan spectra of the reaction between compound III and ascorbic acid. Compound III decays back to native state within 8 s, the scans exhibiting an isosbestic point at 440 nm.

Fig. 6.8 shows stopped-flow traces of the reaction between compound III and ascorbic acid. Pseudo-first order conditions were maintained by using at least 100-fold excess of ascorbic acid. The reaction was followed at 430 nm, corresponding to the formation of the native enzyme. The reaction of ascorbic acid with compound III obeyed first order kinetics. Fig. 6.9 shows the concentration dependence of the pseudo-first order rate constant. From the slope the second order rate

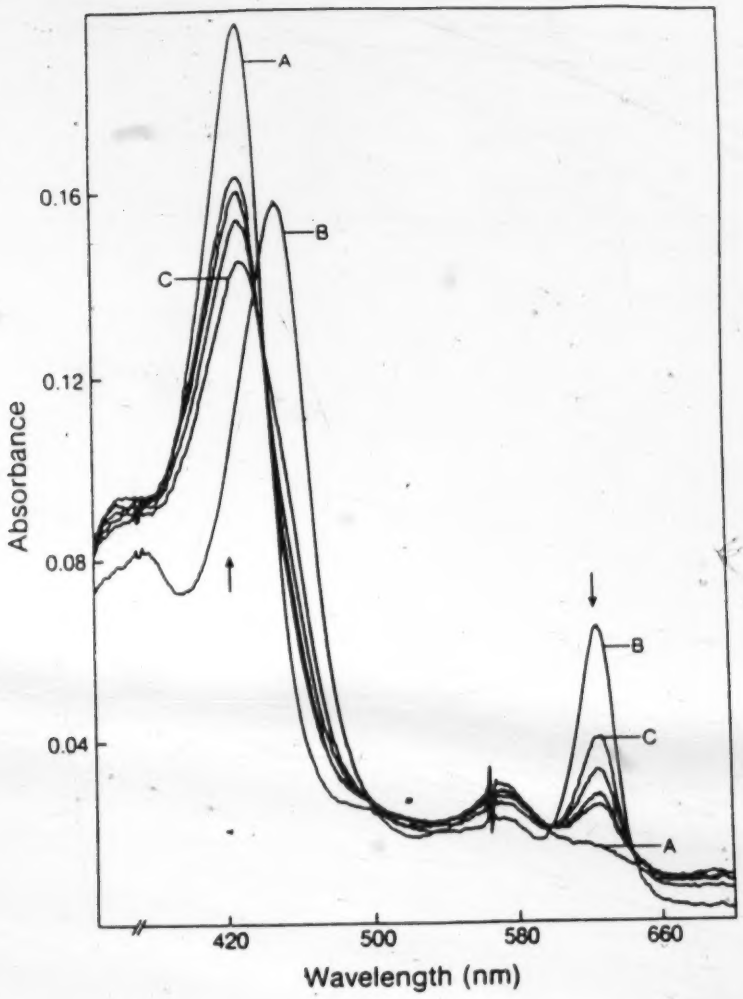

Figure 6.6. Effect of SOD on compound III decay. A) 1.1 μM myeloperoxidase in 0.1 M phosphate buffer, pH 4.7. B) Compound III was formed by adding 100 μM DHF and 0.5 μM H_2O_2 to A. C) 6.5 μM SOD added to B. Arrows show direction of absorbance changes with time.

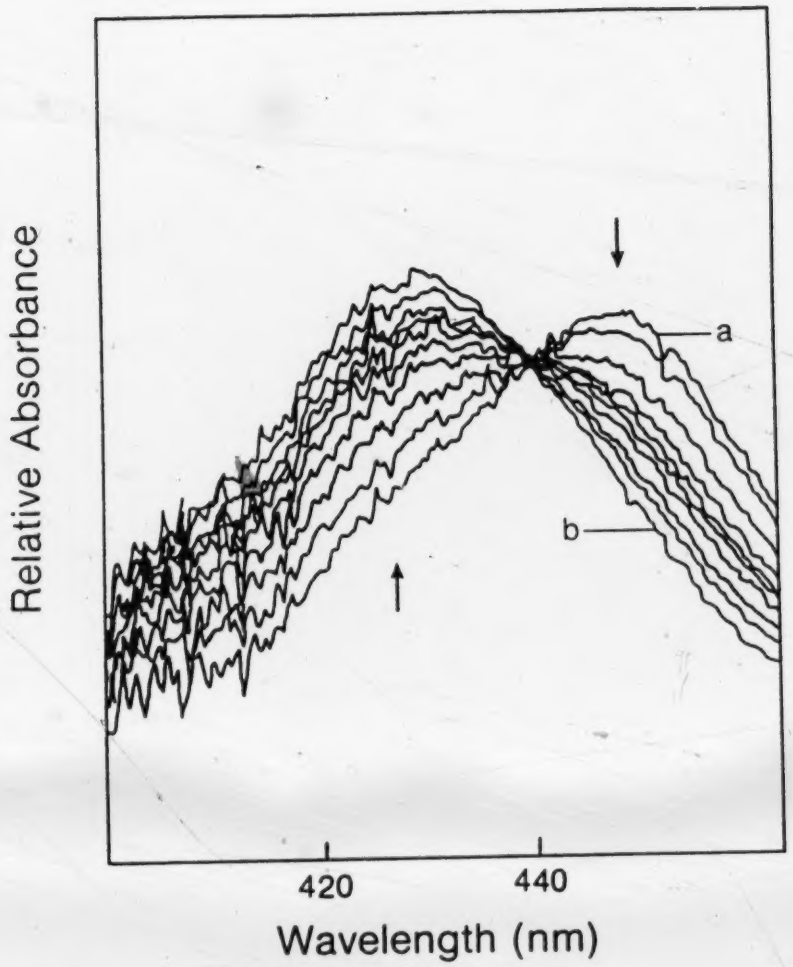

Figure 6.7. Rapid spectral scans of the reaction between compound III and ascorbic acid. One reservoir contained compound III prepared as described in Fig. 6.1, in 0.2 M phosphate buffer, pH 7.1. The other reservoir contained 1 mM ascorbic acid in aqueous solution. Spectra a) was taken 650 ms and b) 8.3 s after mixing.

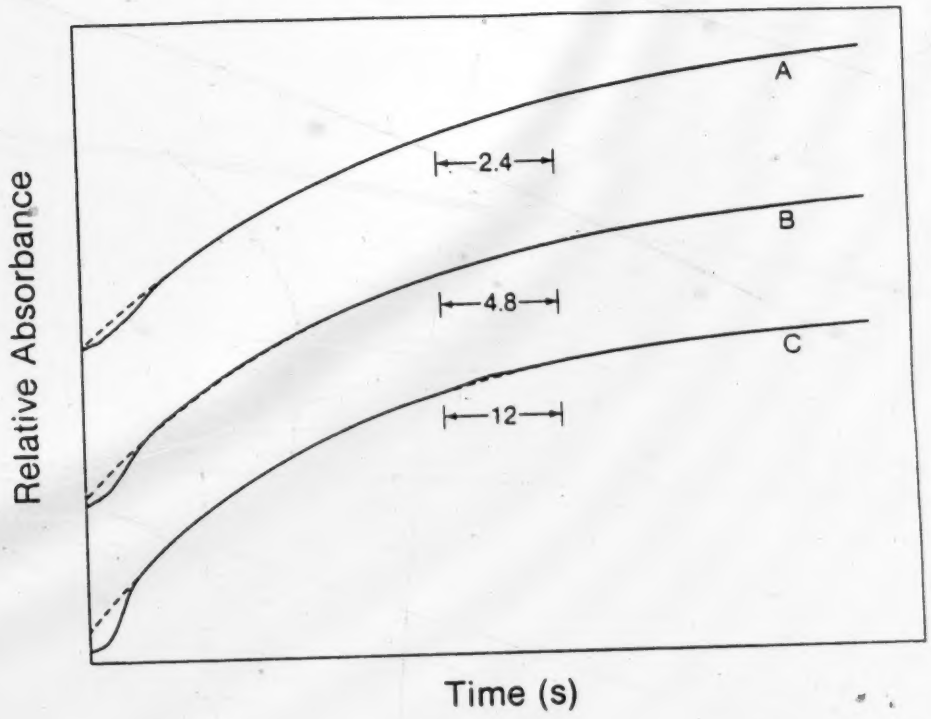

Figure 6.8. Typical time course traces of the reaction between compound III and ascorbic acid. Absorbance changes were followed at 430 nm. One reservoir contained compound III prepared as described in Fig. 6.7 while the other reservoir contained at least 100-fold excess ascorbic acid. A) 0.625 mM; B) 0.250 mM; C) 0.125 mM ascorbic acid The broken line are exponential curve fits.

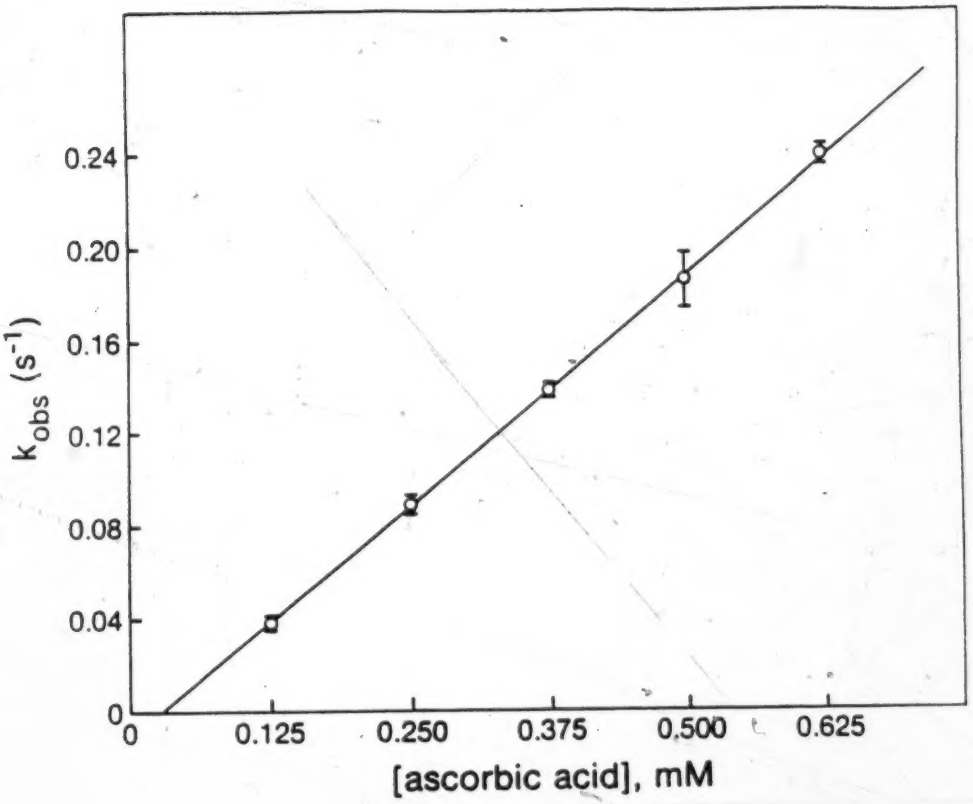

Figure 6.9. Plot of pseudo-first order rate constant (k_{obs}) versus ascorbic acid concentration. Experimental conditions are the same as in Fig. 6.8. k_{obs} was determined from the exponential increase in absorbance at 430 nm. The plot is a linear least squares fit of the data.

constant for the reaction is found to be $(4.0 \pm 0.1 \times 10^{2}) M^{-1}s^{-1}$.

6.4. DISCUSSION

Compound III (also known as oxyperoxidase) is one of the oxidized intermediates of peroxidases. Although it does not participate in the normal peroxidatic cycle its formation, structure and reactivity has nonetheless been of considerable interest. Compound III may be represented as a resonance hybrid between the complexes ferroperoxidase-oxygen and ferriperoxidase-superoxide (i.e. Fe(II)-O_2 <–> Fe(III)-$O_2^{-\cdot}$) (15-18). The formation of compound III can occur through three possible reaction pathways: ferrous enzyme and dioxygen, ferryl enzyme (compound II) and H_2O_2 or ferric enzyme (native) and superoxide (11,19).

In the case of myeloperoxidase, compound III has been prepared through several methods: a) aerobic oxidation of NADH in the presence of 2,4-dichloro-phenol (1); b) direct reaction between ferrous myeloperoxidase and molecular oxygen (1,7); c) addition of a large excess of H_2O_2 to compound II (3); d) addition of $O_2^{-\cdot}$, produced from the electrolytic reduction of molecular oxygen in an aprotic solvent, to native enzyme

(20); and e) direct reaction between ferric myeloperoxidase and $O_2^{-\cdot}$ generated from the xanthine-xanthine oxidase system (18,21).

The reaction between ferric myeloperoxidase and superoxide anion radical has gained a wide degree of attention due to its possible involvement in the enzyme's microbicidal activity. In leucocytes $O_2^{-\cdot}$ production was observed to be enhanced in the presence of latex particles, an increase presumably associated with ingestion of the particles into vacuoles (6). Compound III is reportedly formed in intact phagocytosing granulocytes (7). Earlier studies suggest that myeloperoxidase's bactericidal activity was due to the production of $O_2^{-\cdot}$ accompanying the decay of compound III (22). On the other hand, other authors who studied the same process concluded on the basis of available spectral data that compound II was formed (23). The methods used to prepare compound III have always been plagued with the presence of compound II as an impurity. Moreover, since the Soret spectra of these two intermediates are very similar it is often difficult to discriminate between them.

In an attempt to obtain a relatively pure preparation of compound

III, we generated perhydroxyl radical ($HO_2\cdot$, the protonated form of $O_2^{-}\cdot$) via the aerobic oxidation of DHF in the presence of H_2O_2 and reacted it with ferric myeloperoxidase. This method has been originally applied to HRP (11) and more recently to lignin peroxidase (24). DHF was chosen over the other hydrogen donors because it was found to be almost inactive in reducing compound III to native enzyme (15). In this work, we used bovine spleen myeloperoxidase which has been demonstrated to be identical with granulocyte myeloperoxidase (25).

Two criteria have been proposed to evaluate the purity of compound III preparations: first, symmetry of the Soret band and second, the ratio $A_{625\ nm}/A_{456\ nm}$. For compound II, this ratio is 0.17 whereas for compound III, the value is 0.52. In mixtures, the value lies between these two extremes (3). Fig. 6.1 shows that the first criteria is well satisfied. The sharp peaks at 450 and 625 nm and the broader one at 575 nm are also consistent with the reported spectra of compound III (1,3,26). The ratio $A_{625\ nm}/A_{456\ nm}$ obtained is 0.44, which is better than the values obtained using other methods of preparation (1,3,7,18,20).

Aside from its relative purity, compound III prepared this way was

also found to be very stable as shown by the decay spectra (compare Figs. 6.3 and 6.4). An isosbestic point at 440 nm observed in the decay spectra of both preparations suggests that the conversion from compound III to native does not pass through any other intermediate. However, it is interesting to note in Fig. 6.3 that the initial scans have a different isosbestic point, i.e. at 436 nm. It is possible that the compound II present in the preparation reacts with H_2O_2 formed from the dismutation of $O_2^{-\cdot}$ (Eqn. 6.1) and/or the reaction between $O_2^{-\cdot}$ and DHF (Eqn. 6.2) (27) to give compound III.

[6.1] $$2O_2^{-\cdot} \xrightarrow{H^+} H_2O_2 + O_2$$

[6.2] $$O_2^{-\cdot} + DHF + 2H^+ \longrightarrow DHF^{\cdot} + H_2O_2$$

Thus, initially, the peak at 455 nm (characteristic of compound II) decreases and shifts to 450 nm suggesting compound III formation. As is to be expected, there is very little increase in the absorbance of the native enzyme during this time period. Some workers have proposed earlier that compound III reacts with compound II to give native enzyme (7). The spectral scans shown here do not support this possibility.

Moreover, their proposed reaction requires electron transfer between two states of the enzyme which is not very likely compared to reaction with smaller substrates.

The stability of myeloperoxidase compound III relative to the superoxide adduct of other peroxidases has been documented (7,18). In this work it was found that a greater proportion of native enzyme was converted to compound III at lower pH. Moreover, the compound III formed at lower pH was most stable (Fig. 6.5). It would also be expected that compound III formation will be faster at low pH because $HO_2^{\cdot}$ reacts with the ferric enzyme more rapidly than $O_2^{-\cdot}$ (28,29). Since MPO-catalyzed halogenation has a pH optimum at 5 (30,31), it would seem that the accumulation of compound III at acid pH would limit the microbicidal activity of the enzyme. How then does the phagocytic system prevent this from happening?

Previously it was reported that SOD inhibited DHF oxidation by HRP (32). The results here indicate that myeloperoxidase has a very high affinity for $O_2^{-\cdot}$ because compound III formation was only partially inhibited by up to 3 eq of SOD. When SOD was added after compound III has

been formed the rate of decay to the native form was faster suggesting that SOD is able to scavenge $O_2^{-}\cdot$ from the oxyperoxidase complex.

If less than 100 eq of DHF is added to the enzyme there is incomplete formation of compound III. Considering MPO's strong affinity for $O_2^{-}\cdot$ and that sufficient $O_2^{-}\cdot$ was generated in the system, conversion to compound III should have been quantitative. The possible explanation for these results is based on the finding that compound III has SOD activity (26). It was suggested that the following reaction occurs in the phagocytes.

[6.3] Compound III + $O_2^{-}\cdot$ ——> Compound I + O_2

Moreover, it has been reported that the apparent SOD activity observed in azurophil granules was attributable not to SOD itself but to MPO (33). Evidence of the one-electron reduction shown in Eqn. 6.3 is given by the reaction between compound III and one-electron donors such as p-cresol and ferrocyanide (15). Compound I is the active intermediate in the chlorination reaction. Thus, the reaction shown in Eqn. 6.3 would provide a pathway to ensure that the enzyme is in an active form.

There are other reducing agents present in the phagocyte. Ascorbic acid is present in substantially high concentration (approx. 0.7 mM) (34). In the presence of a large excess of ascorbic acid compound III is rapidly converted back to the native state without formation of any detectable intermediate (Fig. 6.7). Interestingly, the time course traces of the reaction showed a biphasic character (Fig. 6.8). The faster initial phase can be attributed to the reaction between ascorbic acid and compound II (35, see also Chapter 5) inadvertently formed in the preparation (see earlier Discussion). The pseudo-first order rate constants obtained from the second phase of the reaction correspond to the reaction between compound III and ascorbic acid.

Recently, spectral studies on the formation and decay of compound III of canine myeloperoxidase showed that the enzyme could act as $O_2^{-}\cdot$ scavenger and that compound III could generate $O_2^{-}\cdot$ during its decay process (18). Superoxide plays an important role in the formation of H_2O_2 during the phagocytic process. H_2O_2 production was never observed without $O_2^{-}\cdot$ production (36). It was further proposed that $O_2^{-}\cdot$ can also be produced by the oxidation of ascorbate (Eqn. 6.4) (37,38).

[6.4] $AH^- + O_2 \longrightarrow AH\cdot + O_2^{-}\cdot$

The results of this work as well as other documented studies show that ascorbic acid plays an extensive role in the myeloperoxidase system. The primary function of the enzyme is to oxidize Cl^- to the bactericidal agent HOCl. The active enzyme intermediate for this reaction is compound I, which is formed when a stoichiometric equivalent of H_2O_2 reacts with the enzyme. H_2O_2 is produced from the dismutation of $O_2^{-}\cdot$. When a large quantity of $O_2^{-}\cdot$ is generated, dismutation is faster and more H_2O_2 is produced. In such an event, both compounds II (native enzyme + excess H_2O_2) and III (native enzyme + $O_2^{-}\cdot$) may form. The enzyme thus becomes inactive in the chlorination reaction. However, in the presence of reducing agents like ascorbic acid compounds II and III are converted back to the native state and made available for HOCl formation. Thus, the bactericidal activity of the enzyme is maintained.

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CHAPTER SEVEN

GENERAL DISCUSSION

The human body is constantly exposed to microorganisms but it is amazing how it is able to defend itself against their otherwise harmful onslaught. One of the body's defense mechanisms against microbial infection involves the enzyme myeloperoxidase, the topic of this thesis. The white blood cells, neutrophils and monocytes, both contain myeloperoxidase in cytoplasmic granules and this enzyme is discharged during phagocytosis (1). In the presence of H_2O_2 and a halide, myeloperoxidase constitutes a potent cytotoxic system.

Attempts to isolate myeloperoxidase from normal human blood using a combination of published procedures (2,3) were unsuccessful. The major drawback was obtaining a sufficient quantity of outdated human blood.

The discovery of a green heme protein in both bovine and human spleens (4,5) having similar properties as the myeloperoxidase from human blood (6-9) made possible the isolation of enough enzyme for kinetic studies.

In Chapter 1 the purification procedure was given in detail for easy reproducibility in future investigations using bovine spleen myeloperoxidase. A purity number (Rz=0.82) as high as that obtained for crystalline myeloperoxidase (10), a considerable 19% yield as well as a specific activity comparable to that previously obtained are good criteria of the effectiveness of the procedure. Moreover, characterization studies were performed which showed the great similarity of the spleen enzyme to blood myeloperoxidase.

The first characterization criterion used is molecular weight. Literature values range from 130 to 153 kDa for the holoenzyme (11-15). Myeloperoxidase is unique among the other peroxidases in that it contains two hemes per molecule. There have been conflicting results as to the number of subunits in the enzyme (13,15-19). The work by Andrews and Krinsky on reductive cleavage of myeloperoxidase suggests that myeloperoxidase contains two heavy-light dimers joined along their long axes by a single disulfide bond between the two heavy subunits (20).

Determination of the molecular weight of the enzyme using SDS-PAGE gave two values, i.e. 59 kDa and 15.3 kDa corresponding to the

heavy and light subunits, respectively. This is to be expected because the reduction pretreatment involved in this method causes the disruption of the disulfide linkages between subunits and resolves them into single polypeptide chains. Gel filtration is a more gentle method which rarely causes denaturation of labile substances. The molecular weight obtained by this method is quite low (118 kDa) but this can be attributed to the possible interaction between the gel and the highly basic enzyme (pI≈10) (21). Similar retardation of elution has been observed (6) leading to a lower estimated molecular weight.

Cyanide binding was used to investigate further the active site of the enzyme (22). The spectral changes accompanying binding of cyanide to human myeloperoxidase are similar to that of cyanide binding to the spleen enzyme (8). Titration studies further show that the spleen enzyme contains two binding sites per molecule.

The availability of canine myeloperoxidase from a previous collaborative work made possible a comparative study of the equilibria and kinetics of cyanide binding to the same enzyme from a different source. The results are described in Chapter 3. Spectral results support the presence of two heme binding sites; an isosbestic point at 444 nm

and a linear Scatchard plot suggest that the binding affinity of cyanide to the two subunits of the enzyme is the same. The dissociation constant 0.53 μM shows that cyanide has a very strong affinity for the enzyme. In fact, compared with the other peroxidases, myeloperoxidase exhibits the strongest affinity for cyanide and this is probably also due to its unique chlorin prosthetic group (23-26). The pH dependence of the apparent second order rate constant indicates the presence of an acid-base group on the enzyme with a pKa of 3.8 ± 0.1. The protonated form of cyanide binds to the basic form of the enzyme. This is also true of other peroxidases (27-29).

Further characterization of the isolated spleen enzyme was done by studying the peroxidase intermediates compounds I and II (Chapter 4). Full formation of compound I required that about 20-fold excess of H_2O_2 be added to the native enzyme. A lower excess led to incomplete compound I formation possibly due to the catalatic activity of the enzyme (30). Compound I decays very fast to compound II and thus, in the absence of a multi-mixing stopped flow apparatus, transient state kinetic experiments employing compound I were not done. Compound II is more stable and easily prepared. Compound II decays back to the native

state without passing through any intermediate. The H_2O_2 necessary for the formation of these intermediates probably comes from the dismutation of $O_2^{-}\cdot$. Phagocytosis was always found to be accompanied by a respiratory burst and oxygen consumption is attributed to the reduction of O_2 to $O_2^{-}\cdot$ by NADPH oxidase (31-36).

All these analogous results in molecular weight, number of subunits, cyanide binding properties and spectroscopic properties of intermediates between blood myeloperoxidase and the spleen enzyme provide considerable evidence that the two enzymes are similar if not identical.

Because of the great similarity of the spleen and blood enzymes it is likely that their physiological roles are also similar if not identical. This statement is consistent with the physiological role of the spleen. One of the functions of spleen is to clean the system of old and damaged blood cells and foreign matter. Large phagocytic white cells (monocytes) are present in great numbers in the splenic pulp (37). Myeloperoxidase isolated from blood monocytes (38) is similar to neutrophil myeloperoxidase. The spleen is therefore a better source of the

enzyme because this organ contains a tremendous number of phagocytic cells. Moreover, it serves as a sink for worn out myeloperoxidase-containing neutrophils.

While various mechanisms have been proposed to explain the cytotoxicity of the myeloperoxidase-H_2O_2-Cl^- system (see Chapter 1), the common theme is the initial generation of HOCl. Compound I is the intermediate which oxidizes Cl^- to HOCl while compound II is inactive in the chloride peroxidation reaction (39). It is therefore important to keep a pool of native enzyme available for compound I formation and yet avoid the accumulation of compound II. Ascorbic acid is known to stimulate leucocyte functions. In a recent publication it was suggested that the role of ascorbic acid is to reduce compound II of myeloperoxidase back to the native enzyme (40). The results presented here are consistent with this hypothesis. In Chapter 5 rapid spectral scan and transient state kinetic results on the reaction of three myeloperoxidase compounds II namely, human neutrophil myeloperoxidase, canine myeloperoxidase and bovine spleen heme protein, with ascorbate were presented. The rapid scan spectra show that compound II does not pass through any other intermediate when ascorbic acid reduces it back to the native form. The

reactions of all three compounds II involve a simple binding interaction before enzyme reduction. The optimum pH is 4.5 and this is consistent with the optimal pH for phagocytic activity (41). Results of this work provide further evidence that the spleen green heme protein is very similar to leucocyte myeloperoxidase based on a comparison of spectral scans, pH rate profiles and kinetic parameters. It was also demonstrated that chloride cannot reduce compound II whereas iodide reduces compound II to native enzyme at a rate comparable to that of ascorbate. This explains why ascorbate accelerates chlorination but inhibits iodination. Formation of compound II is a dead end for the generation of HOCl; ascorbate regenerates more native enzyme to enhance the chlorination reaction namely: myeloperoxidase + peroxide —> compound I; followed by compound I + chloride —> HOCl. On the other hand, ascorbate is a competitor with iodide for both compounds I and II and so inhibits iodination.

Compound III, which can be formed by the reaction of native enzyme with $O_2^{-\cdot}$ is also inactive in chloride peroxidation. Thus, the accumulation of compound III also limits the enzyme's bactericidal

activity. One difficulty in studying compound III reactions however is that its preparation is always plagued by a contamination of compound II.

In Chapter 6, a procedure for preparing a relatively pure and stable compound III of bovine spleen myeloperoxidase was described. It was found that the compound III produced when $O_2^{-\cdot}$ was generated using the oxidation of dihydroxyfumarate was more stable than when the $O_2^{-\cdot}$ was generated using the more popular xanthine-xanthine oxidase system (42,43). Spectral scans show well- defined peaks at 450 and 625 nm and an isosbestic point with the native enzyme at 440 nm. Compound III decayed to native enzyme without any detectable intermediate. The rate of decay was faster at pH above neutral and also in the presence of superoxide dismutase. Ascorbic acid is also capable of reducing compound III to the native form.

Figure 7.1 summarizes the possible role of ascorbic acid in the chloride peroxidation reaction catalyzed by myeloperoxidase. The primary function of the enzyme is to oxidize chloride to the bactericidal agent HOCl. The active enzyme intermediate for this reaction is compound I. However, in the presence of excess H_2O_2 and $O_2^{-\cdot}$ both

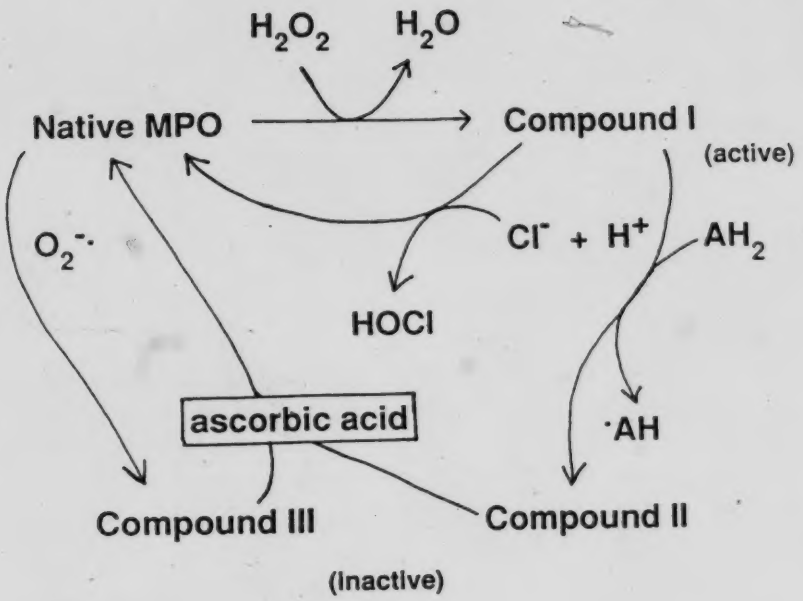

Figure 7.1. Schematic representation of the possible role of ascorbic acid in the chloride peroxidation reaction. Compound I is the active intermediate. The formation of inactive compounds II and III limits the microbicidal activity of the enzyme. Ascorbic acid reduces these intermediates back to the native state and thus, makes them available for HOCl formation.

compounds II and III form, which are inactive in the chlorination reaction. Thus, the accumulation of these intermediates limits the microbicidal activity of the enzyme. Ascorbic acid reduces compounds II and III back to the native enzyme and makes it available for HOCl formation. Thus, the bactericidal activity of the enzyme is maintained.

SUGGESTIONS FOR FUTURE WORK

The transient state kinetic studies done in this work involved the more stable intermediates of bovine spleen myeloperoxidase, namely, compounds II and III. Compound I is the active intermediate for the halide peroxidation reaction. It is therefore of tremendous significance to study the various substrates and factors which directly affect this reaction system. Unfortunately, compound I is a very short-lived species such that conventional stopped flow technique cannot be used. An alternative approach would be the use of a multi-mixing apparatus wherein compound I can be reacted very fast with the appropriate substrates.

In this work it was shown how ascorbic acid stimulates chloride peroxidation by converting the inactive compounds II and III back to the native enzyme. Glutathione is another reducing substrate ubiquitous in

the human system. Its effect on HOCl generation could likewise be investigated. Moreover, the mechanism of one or two-electron oxidation of glutathione can be studied as was done with lactoperoxidase and thyroid peroxidase (44).

Myeloperoxidase has not only been implicated in antimicrobial host defense but deleterious injuries to host structures as well. For example, catechols and catecholamines can function as electron donor substrates for myeloperoxidase. Oxidative degradation of these substances has been a current research interest due to their cytotoxic products. Recently, evidence for peroxidatic oxidation of norepinephrine, a catecholamine, by lactoperoxidase was presented (45). Similar metabolites accumulate in the bone marrow, which contains high levels of myeloperoxidase (46). Thus, a study of myeloperoxidase-catalyzed oxidation of catechols and catecholamines could lend insight into the mechanisms underlying the toxicity induced by aromatic compounds which yield these secondary phenolic metabolites (47).

Another area which is open for investigation is myeloperoxidase-oxidase reactions which are characterized by their use of O_2 instead of H_2O_2 as external cosubstrate for the oxidation of compounds such as thiols (48).

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APPENDIX

DERIVATIONS

All kinetic experiments described in this thesis were performed under pseudo-first order conditions. The second order kinetic equation can be derived by considering the reversible formation of a complex, ES, between enzyme, E, and substrate, S :

$$[1] \qquad E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

This system follows second order kinetics where:

$$[2] \qquad k_{obs} = k_1 [S] + k_{-1}$$

Two approaches will be used to derive Eq. [2].

A) Relaxation Theory Approach

Starting with the rate equation:

$$[3] \qquad -d[E]/dt = k_1[E][S] - k_{-1}[ES]$$

At equilibrium, $-d[E]/dt = 0$ and Eq. [3] becomes

[4] $k_1[E]_{eq}[S]_{eq} = k_{-1}[ES]_{eq}$

When this equilibrium state is perturbed, the system adjusts itself and this is usually manifested by a measurable change in the concentrations of the species partaking in the equilibrium. If we denote the perturbation by Δ, the following conservation relations can be written:

$$[E] = [E]_{eq} + \Delta[E]$$

$$[S] = [S]_{eq} + \Delta[S]$$

$$[ES] = [ES]_{eq} + \Delta[ES]$$

Expressing these relations in terms of $\Delta[E]$, then $\Delta[E] = \Delta[S] = -\Delta[ES]$:

[5] $[E] = [E]_{eq} + \Delta[E]$

[6] $[S] = [S]_{eq} + \Delta[E]$

[7] $[ES] = [ES]_{eq} - \Delta[E]$

The rate equation can now be written as:

$$-d[E]/dt = -d([E]_{eq} + \Delta[E])/dt$$

But since $[E]_{eq}$ is a constant, $-d[E]/dt = -d\Delta[E]/dt$

Substituting Eqs. [5] to [7] to Eq. [3]:

$$[8] \quad -d\Delta[E]/dt = k_1([E]_{eq} + \Delta[E])([S]_{eq} + \Delta[E]) - k_{-1}([ES]_{eq} - \Delta[E])$$

$$= k_1[E]_{eq}[S]_{eq} + k_1[E]_{eq}\Delta[E] + k_1[S]_{eq}\Delta[E] + k_1\Delta[E]^2$$

$$- k_{-1}[ES]_{eq} + k_{-1}\Delta[E]$$

If the perturbation is very small, $\Delta[E]^2$ will be negligible. From Eq. [4], the first and fifth terms cancel.

$$[9] \quad -d\Delta[E]/dt = k_1[E]_{eq}\Delta[E] + k_1[S]_{eq}\Delta[E] + k_{-1}\Delta[E]$$

$$= \{ k_1([E]_{eq} + [S]_{eq}) + k_{-1} \}\Delta[E]$$

If we define $-d\Delta[E]/dt = (1/\ )\Delta[E]$

$$[10] \quad 1/\ = k_1([E]_{eq} + [S]_{eq}) + k_{-1}$$

If [S] is in large excess, $[S]_{eq} >> [E]_{eq}$, $[E]_{eq}$ can be neglected and $[S]_{eq} = [S]_o$.

$$1/\ = k_{obs} = k_1[S] + k_{-1}$$

B) Conventional kinetic theory approach

Consider the rate equation [2] above. If the total substrate concentration,

$[S]_o$, is much greater than the total enzyme concentration, $[E]_o$, the former can be assumed to remain constant during the course of the reaction. The reaction now becomes pseudo-first order and the rate constant $k_1' = k_1[S]$. Eq. [2] becomes:

[11] $-d[E]/dt = k_1'[E] - k_{-1}[ES]$

At equilibrium, this leads to:

[12] $k_1'[E]_{eq} = k_{-1}[ES]_{eq}$

The following conservation relations can also be written:

$[E]_o = [E] + [ES]$ and therefore,

[13] $[ES] = [E]_o - [E]$ and $[ES]_{eq} = [E]_o - [E]_{eq}$

Substituting [13] to [11] and rearranging,

[14] $-d[E]/dt = (k_1' + k_{-1}) \{ [E] - k_{-1} [E]_o/(k_1' + k_{-1})\}$

Substituting [13] to [12] and rearranging,

[15] $[E]_{eq} = k_{-1} [E]_o/ (k_1' + k_{-1})$

Eq. [14] becomes, upon substitution of [15],

[16] $-d[E]/dt = (k_1' + k_{-1})([E] - [E]_{eq})$

$-d[E]/([E] - [E]_{eq}) = (k_1' + k_{-1})\,dt$ which leads to:

[17] $\ln ([E]_o - [E]_{eq})/([E] - [E]_{eq}) = (k_1' + k_{-1})\,t$

$k_{obs} = k_1' + k_{-1}$ and $k_1' = k_1[S]$

Thus,

$$k_{obs} = k_1[S] + k_{-1}$$

which is identical in form as Eq. 3.1.

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